5-2012

Sanitation Assessment of Food Contact Surfaces and Lethality of Moist Heat and a Disinfectant Against Listeria Strains Inoculated on Deli Slicer Components

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SANITATION ASSESSMENT OF FOOD CONTACT SURFACES AND LETHALITY OF MOIST HEAT AND A DISINFECTANT AGAINST *LISTERIA* STRAINS INOCULATED ON DELI SLICER COMPONENTS

SANITATION ASSESSMENT OF FOOD CONTACT SURFACES AND LETHALITY OF MOIST HEAT AND A DISINFECTANT AGAINST *LISTERIA* STRAINS INOCULATED ON DELI SLICER COMPONENTS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

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May 2012
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ABSTRACT

The overall objectives of this study were to: evaluate the efficacy of different cleaning cloth types and cloth-disinfectant combinations in reducing food contact surface contamination to acceptable levels; determine the optimum moist heat and moist heat + sanitizer treatments that can significantly reduce the number of *Listeria* strains on deli slicer components; and investigate if the moist heat treatment used in this study induced the viable-but-non-culturable (VBNC) state in *Listeria* cells. The efficacy of wiping cloths was measured using ATP-bioluminescence and total plate count methods using four different wiping cloths and silver dihydrogen citrate sanitizer on food contact surfaces. The lethality study of moist heat and silver dihydrogen citrate disinfectant against *Listeria* strains was done using deli slicer components and the viable-but-non-culturable state of *Listeria* strains subjected to sub-lethal moist heat and silver dihydrogen citrate disinfectant stresses was measured using BacLight bacterial viability test kit. In the first study we demonstrated that the cleaning effects of wiping cloths on food contact surfaces can be enhanced when used with the SDC sanitizer and stated that the ATP-B measurements can be used for real-time hygiene monitoring in the public sector with inclusion of microbial contamination testing (total plate count) for more reliable measure of cleanliness. In the moist heat lethality study, the internal moist heat only treatment and both the internal and external moist heat + disinfectant treatments yielded non-detectable levels of *Listeria* strains on stainless steel and cast aluminum coupons. Moist heat only and moist heat + disinfectant treatments at 150 °F (66°C) and at least 20% relative humidity (RH) for 5 h was adequate to attain non-detectable levels of a *Listeria* strains cocktail on both stainless steel and cast aluminum deli meat slicer components. The BacLight bacterial viability test demonstrated that the moist heat treatment applied in this study was effective in inactivating *Listeria* strains. However, the absence of growth on nutrient agar plates and detection of live cells by the viability test demonstrated that the sub-lethal temperature used in this study could induce the VBNC state in *Listeria* strains.

This thesis is approved for recommendation

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ACKNOWLEDGMENTS

I am exceedingly indebted to my advisor, Dr. Philip G. Crandall for willing to accept me as his student and for providing me this chance and inspiration for this research. His overall direction, guidance and tolerance for my familial obligations have not gone unnoticed. Dr Elizabeth M. Martin, your enormous capacity for guiding, motivating and encouraging me throughout the course of my research has been amazing! I still can't put my fingers on how you do it…with so much work in your hands and so many students who need your help, you were able to somehow "multitask" and help us whenever we needed you. I cannot forget to thank Dr. Dinesh Babu for stepping in at the right time and guiding me throughout my research with his expertise…you pushed me hard, but it was worth it! I also wish to thank my committee members: Dr. Steven C. Ricke, Dr. Navam S. Hettiarachchy, and Dr. Jean-François Meullenet, who were more than generous with their expertise and precious time as well as for agreeing to serve on my committee. Last but not least, I would like to extend my sincere gratitude to the Fulbright Scholarship Program for funding my studies in the USA; the Institute of International Education, particularly Emily Bosio, and the University of Arkansas Sponsored Students Program (Gloria Flores, Catherine Cunningham and their team) for their dedication to international sponsored students support. It's been a long roller coaster ride but I finally made it!

DEDICATION

I dedicate this work and give special thanks to my wife and best friend, Sene, as well as our two wonderful kids Wami and Yenzo for their understanding and patience while I was away from them. You have all been my inspiration!

A special feeling of gratitude goes to my loving mother, Juliet K. Masuku who did all she could for me to get to where I am today even before I knew I needed to go this far. Her words of encouragement and tenacity still echo in my ears. To my sisters and brother, guys thanks for being there as family and may God bless you exceedingly…you are very special.

I also dedicate this thesis to my many friends out there who have supported me throughout the duration of my studies. Mduduzi Dlamini, I still recall the day you walked into my office to invite me to the interview that ended up bringing me here…God used you! Felix Masuku (Mnaketfu!), Mbulelo Dlamini (S'khutshwa), Andrew Dlamini and your families, thank you for teaching me what friendship is all about!

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CHAPTER I

GENERAL INTRODUCTION

Statement of the problem

Food handling facilities provide a suitable environment for microbial growth (Evans *et al.* 1998, Tebbutt 1984). The organic materials handled within food-service establishments and the food contact surfaces harbor food pathogens, and any inadequate cleaning and disinfection of food contact surfaces, therefore, present high risks for cross contamination (Moore and Griffith 2002). The food contact surfaces in food-service facilities include, food preparatory tables, benches, both cutlery and crockery, serving tables and deli meat slicers. The main concern on contaminated surfaces is the potential presence of pathogens particularly those with a low infective dose and those that are known to cause food borne disease outbreaks such as listeriosis caused by *Listeria monocytogenes*. *L. monocytogenes* is persistent and has been reported to inhabit food-service establishments for very long periods even at refrigeration temperatures creating problems in its control. Donganay (2003) has reported that even though foodborne illnesses caused by *L. monocytogenes* are rare, the fatality rate of 29% is one of the highest among foodborne pathogens in immuno-compromised individuals. Based on the report by Mead *et al.* (1999) which estimated foodborne listeriosis cases at 5,205 per year in the USA, Scharff (2010) estimated the total cost per listeriosis case to be $1,695,143 which translates to a total cost of $8,823 million for all foodborne listeriosis cases per year in the USA alone. Collins (1997) has reported that approximately 80% of reported foodborne outbreaks have been traced to food-service facilities where cross contamination has been reported as a major contributing factor. Several outbreaks have been linked to deli meat slicers in the retail deli environment. Gombas *et al.* (2003) has also demonstrated that ready-to-eat (RTE) deli meats sliced at retail stores have a seven times higher prevalence of *Listeria monocytogenes* contamination compared with prepackaged RTE products.

Study justification

A significant reduction in bacterial load and improved hygiene conditions in food-service establishments can be achieved by use of efficient cleaning cloths and sanitizers. Tebbutt (1984) reported that cleaning and rinsing alone may achieve a 2 to 3 log reduction. Crandall *et al.* (2012) demonstrated that sanitizers were able to remove 2 to 3 log CFU/cm^2 of inoculated *Listeria* strains attached on food contact surfaces of stainless steel. It has also been demonstrated that moist heat is much more effective in reducing bacterial load than dry heat (Crandall et al. 2010, Murphy *et al*. 1999, 2001) and Crandall *et al.* (2009) have reported that subjecting deli slicer components to 82°C for 3 hours under saturated moisture conditions could produce a 5 log reduction of the more heat resistant *Listeria innocua*. The use of deli bread proofer ovens has been recommended by Crandall *et al.* (2010) to provide the moist heat conditions required to sanitize deli meat slicers because most deli outlets own one. Several heat inactivation studies have, however, revealed that bacteria, including *Listeria monocytogenes*, exposed to sub-lethal heat may convert to a viable-but-not-culturable (VBNC) state in an effort presumably to cope with the prevailing conditions (Boulos *et al.* 1999, Li *et al.* 2003, Lindback *et al.* 2010). The VBNC state of pathogenic organisms is feared to be a threat to public health because cells in this condition cannot be detected with the conventional culturing methods yet they are still viable and are resuscitated when the conditions become favorable (Byrd *et al.* 1991). Thus, apart from routine cleaning and sanitization, regular monitoring of the effectiveness of cleaning regimes could serve as a crucial preventative measure for potential foodborne outbreaks. For the periodic microbiological assessment of high-risk food service establishments, it is necessary to use testing methods that will give immediate feedback so that appropriate preventative action can be taken before cross-contamination can occur. In this study, we provided data on effectiveness of cleaning materials and correlated the adenosine triphosphate bioluminescence (ATP-B) and total plate count (TPC) methods. Furthermore, the efficacy of the bread proofer oven to provide adequate moist heat for aiding in sanitizing deli meat slicers in addition to chemical sanitizer usage, along with estimation of % live cells using the Live/Dead BacLight viability test

were conducted to determine the ability of the evaluated *Listeria* inactivation methods on the treated food contact surfaces.

Study objectives

This study intended to: assess the effectiveness of selected cleaning cloths in cleaning food contact surfaces; determine the effectiveness of cloth-sanitizer combinations in reducing food contact surface contamination to acceptable levels; and determine if the detection capability of the two testing methods (ATP-B, and TPC) is the same. It also aimed at determiniining the optimum moist heat treatment and moist heat and sanitizer combination necessary to significantly reduce *Listeria* strains on deli slicer components and to rule out the presence of VBNC cells after the moist heat treatment.

Context of the study

This study drew upon research conducted as part of a comprehensive project funded in part by KALLE USA, the American Meat Institute Foundation and by the United States Department of Agriculture's (USDA) National Integrated Food Safety Initiative (NIFSI, grant number 2010-51110-21004). The main project aimed at investigating cost effective treatments to minimize *Listeria monocytogenes* cross-contamination of ready-to-eat meats by the retail deli meat slicer. The primary goals of the main study were to develop a visual verification system to insure that food contact surfaces are "clean to sight and touch"; improve cleaning and sanitation methods to insure their effectiveness in removing *Listeria* and *Listeria* biofilms; assess the effectiveness of "hot boxes" or deli bread proofer ovens to sanitize clean slicers overnight; and to draft "Best Practices" and test under commercial conditions for cleaning and giving deli meat slicers a lethal heating step. This study, therefore, addressed a part of these objectives.

Research question and hypothesis

In view of the adverse consequences of cross-contamination in retail food-service facilities, it is imperative to determine appropriate cleaning and sanitizing materials and methods which can be used in these facilities to improve hygiene and subsequently public health. The research questions to which this study focused were:

1. "Which cloth type and cloth-sanitizer combination is more effective in reducing contamination from food contact surfaces?"
2. "Is it possible to achieve a 5 log reduction of a cocktail of *Listeria* strains, inoculated on coupons cut from deli slicer components, by subjecting them to a sanitizer and moist heat in a deli bread proofer oven, at 150°F for 5h and 7h?"
3. "Do *Listeria* strains convert to the viable-but-non-culturable state when subjected to 150°F for 7h and to silver dihydrogen citrate?"

We hypothesized that:

1. "The efficacy of the cloth types and cloth-sanitizer combinations, in reducing contamination from food contact surfaces, will differ."
2. "A combination of moist heat at 150°F for 5h and 7h, in a bread proofer oven, and a sanitizer can achieve a 5 log reduction of a cocktail of *Listeria* strains, inoculated on coupons cut from deli slicer components."
3. "*Listeria* strains will convert to the viable-but-non-culturable state when subjected to 150°F for 7h and to silver dihydrogen citrate."

Assumptions

This study was based on the following assumptions:

1. The food-service facility staff used all the test cloths, supplied to them in the first study, in a similar manner as they did on a day-to-day basis.
2. The experimental units (Formica table surfaces), in the second cloth study, had all been used, and as such dirty, prior to taking positive control samples.
3. The internal part of the motor compartment (MC) of the deli slicer represents the "cold spot" in the deli slicer and that it is the last to reach the oven proofer temperature. This area may also represent the hard to reach niches of the slicer.

4. The brain heart infusion (BHI) broth, in which the *Listeria* cocktail is grown and kept in suspension, provides some protection to *Listeria* cells just like food debris would in the hard to clean areas of the deli slicer.

Limitations

1. Only one type of food contact surface (Formica table surface) was used in both the first and second cloth studies, which limits the results of the study to this type of surface only.
2. The surfaces, in the second cloth study, had to be wiped with a clean cotton terry bar cloth, after being sprayed with the sanitizer, to minimize the dampness on the sprayed surfaces before taking samples.
3. The cocktail of *Listeria* strains inoculated on coupons was mixed with BHI broth and not with a typical food matrix which would have represented a real life situation where pathogens are protected by food fragments including fat.
4. It was not possible to create a representative harborage such as seams and seals or other hard-to-reach areas which may accumulate liquids and food debris as well as harbor *L. monocytogenes* and other pathogens. The internal part of the MC may not represent all these areas because some of them may not keep moisture as the MC does.

Organization of the study

This thesis contains four chapters. Chapter 1 introduces the need for the study and clearly shows the link between the two studies presented in this thesis. It further presents the purpose, justification, context, research questions and hypotheses, assumptions, and limitations of both studies. Chapter 2 is the literature review of studies addressing cleaning materials, sanitizers and methods used to test contamination on food contact surfaces, as well as *Listeria*, moist heat inactivation of *Listeria* and the viable-but-non-culturable state of *Listeria monocytogenes*. Chapter 3 and 4, respectively, provide a description of both studies focusing on introduction, materials and methods, data analysis, study results, discussion and conclusion.

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CHAPTER II

REVIEW OF LITERATURE

Contamination of food contact surfaces

Food surfaces are essential in the attachment and buildup of foul material composed of microorganisms, soil and other organic materials (Whitehead *et al.* 2010). In the food-service environment, food debris and microorganisms often contaminate food contact surfaces and create problems regarding cleaning and hygiene. The accumulation of food debris on food contact surfaces may lead to the formation of conditioning films – the initial stage in biofilm formation. However, a "true" biofilm is not likely to form on open food contact surfaces because they do not normally provide a suitable environment for microbial attachment (Welker *et al.* 1997, Whitehead *et al.* 2010). Organic matter on food contact surfaces can provide a good source of nutrients for the growth of microorganisms including foodborne pathogens. Upon attachment to food contact surfaces, bacteria produce extracellular polymeric substances and other particulate materials and to bind together forming biofilm (Whitehead *et al.* 2010). Contamination of foods in the food industry has been reported to predominantly come from hands or via contaminated food contact surfaces and equipment (Tebbutt *et al.* 2007). Effective and regular cleaning of food contact surfaces is therefore imperative particularly in the food industry.

Significance of sanitary food contact surfaces

Cross-contamination of food contact surfaces is a major safety concern for food-service facilities where any inefficient cleaning could spread harmful bacteria and viruses posing serious health hazards to consumers. Nearly 80% of the reported foodborne outbreaks can be traced back to food-service facilities with major contributing factors being time/temperature abuse of prepared foods, poor personal hygiene of the food preparer, and cross contamination in the food-service establishments (Collins 1997). Thus, microbiological quality evaluation of commercial food preparation and serving surfaces in food-service

establishments is critical to public health. A significant reduction in bacterial load and improved hygiene conditions of such areas can be achieved by use of efficient cleaning cloths and sanitizers. Regular monitoring for the effectiveness of cleaning practices could serve as a crucial preventative measure for potential foodborne outbreaks. From the point of food production and preparation to consumption there could be several opportunities for food to become contaminated. Food contact surfaces have been predominantly incriminated in the contamination of food products in the food industry (Evans *et al*. 1998, Tebbutt 1984). Although cleaning is regularly practiced in food-service establishments, there is great reliance upon visual assessment for hygiene monitoring, which is not reliable in determining the potential risk that contaminated food contact surfaces may constitute (Tebbutt *et al.* 2007). Consequently, the data pertaining to the presence of foodborne pathogens on surfaces of food-service establishments and their potential to spread from surfaces to food is justification enough to ensure effective cleaning regimes. Microbiological assessment of food contact surfaces is therefore necessary to determine the effectiveness of cleaning methods on surfaces (Verran *et al.* 2002).

Effects of food contact surfaces on cleaning efficacy

Several studies have been conducted to assess the effectiveness of cleaning methods on different types of food contact surfaces such as Formica, wood, plastic, glass, stainless steel surfaces among others (Hong and Brown 2009, Tebbutt. 1988, Welker *et al.* 1997). Individual surfaces have been shown to respond differently to different cleaning methods. Glass surfaces have been proven to increase the adhesion of both *Escherichia coli* and *Bacillus brevis* onto the surface resulting in enhanced metabolic activity, assessed through adenosine triphosphate (ATP) bioluminescence assay. The degree of attachment determines the ease or difficulty of the removal of bacterial cells from the surfaces. This phenomenon may result in those surfaces, which allow a high degree of attachment, to reduce the ease of removal by cleaning methods and vice versa (Hong and Brown 2009). Welker *et al.* (1997) demonstrated that wooden surfaces retained more microorganisms than plastic surfaces. They concluded that the difficulty in cleaning wood surfaces, combined with their ability to absorb more moisture resulting in the absorbance of contaminating bacteria, make wooden surfaces less desirable for use as food contact

surfaces. Older and worn out plastic surfaces were reported to reduce the effectiveness of cleaning methods. Stainless steel and Formica are the most preferred and widely used food contact surface in the food-service environment (Tebbutt 1988, Whitehead *et al*. 2008).

Microbiological monitoring methods

Some studies have recommended periodic microbiological assessment of high-risk food establishments to reduce the risk of foodborne disease outbreaks (Moore and Griffith. 2002). The efficacy of sampling methods for evaluating the cleanliness of food contact surfaces has also been extensively studied (Chen and Godwin 2006, Corbitt *et al.* 2000, Griffith *et al.* 2000, Hong and Brown 2009, Larson *et al.* 2003, Matticka *et al.* 2003, Lappalainen *et al.* 2000, Leon and Albrecht 2007, Park and Cliver 1996, Whitehead *et al.* 2008) and the ATP-B assay is one of the widely used methods for the detection of microbial contamination and food residues in the food industry (Chen and Godwin 2006, Corbitt *et al.* 2000, Lappalainen *et al.* 2000, Larson *et al.* 2003, Powitz 2007). Microbiological methods such as the aerobic plate count take up to 48 hours to yield feedback on findings (Leon and Albrecht 2007). ATP is the energy transfer molecule produced by all living biological cells whose presence in a sample is an indication of biological activity. This method operates on the basis of this understanding and uses ATP to determine the availability of viable cells in a sample (Griffith *et al.* 2000). Briefly, the combined use of the enzyme luciferase and ATP bioluminescence in a luminometer creates a reaction that produces light. The quantity of light is expressed in relative light units (RLU), which directly correlates with the amount of ATP present and consequently the amount of organic material on the sampled area (Larson *et al.* 2003). An ATP free buffer, water or extractant can be used to moisten the ATP swab to facilitate the release of ATP from the surface being sampled depending on the type of ATP meter in use. The swabs can then be tested immediately using a portable luminometer (Powitz 2007). Conflicting findings have been reported with regard to the correlation between ATP bioluminescence and the number of bacteria in a sample tested using the TPC method (Tebbutt *et al.* 2007, Whitehead *et al.* 2008). The ATP-B assay is used as an indicator of total cleanliness and, unlike the TPC method, not only gives the number of microorganisms in a sample, but also the number of eukaryotic cells present in a sample. This is

elucidated by the study of Whitehead *et al.* (2008) who reported that ATP bioluminescence assay is poorly correlated to the number of bacteria in a sample from surfaces in a fish processing factory. The presence of food debris indicates that a food contact surface has not been adequately cleaned and may contain pathogenic or harmful materials since food residues are nutrients for the consequent growth of microorganisms (Leon and Albrecht 2007). ATP bioluminescence provides a real-time estimate of total surface cleanliness including the presence of organic debris and microbial contamination (Leon and Albrecht 2007). This should explain the reason why some studies have found poor correlation between ATP results and the number of bacteria in samples. Some studies have revealed that there is a possibility for the ATP luminescence measurement to be significantly affected by cleaning agents and disinfectants. They reported that since the ATP bioluminescence is based on the presence of an enzymatic reaction (luciferase enzyme); the disinfectants can reduce the activity of the luciferase enzyme and also act as ATP-releasing agents, which may give false results (Lappalainen *et al.* 2000). Consideration of the testing environment, specifically the effect of sanitizers and other chemicals has therefore been strongly suggested (Powitz 2007).

Effectiveness of cleaning materials

The choice of both cleaning materials and sanitizers is essential in ensuring hygienic food contact surfaces (Diab-Elschahawi *et al.* 2010). Cleaning methods should sufficiently reduce both food fragments and microorganisms from the surfaces to levels that will pose less or no threat to the product's safety and quality (Moore and Griffith 2002). Even though cleaning cloths and sanitizers are an important part of the cleaning process, reusable cleaning cloths usage is discouraged because of their ability to re-contaminate surfaces; however, food premises have continued to use them (Tebbutt 1991). There is, nonetheless, very little information on the effectiveness of commonly used cleaning materials in the deli environment. Studies have been conducted to determine the effectiveness of cleaning regimes (Griffith *et al.* 2000, Tebbutt 1984, Tebbutt 1991, Worsfold and Griffith 2001) but, with little focus on the efficacy of individual cleaning cloths.

Effectiveness of sanitizers

The use of cleaning cloths in combination with disinfectants has also been investigated (Tebbutt 1991, Tebbutt *et al.* 2007). Sanitizers are used to reduce the number of potential pathogens on food contact surfaces and they can ensure food safety if properly used. Surfaces cleaned with a cloth soaked in a sanitizer are more likely to be successfully cleaned than those wiped with cloths which are not disinfected after use (Tebbutt 1991). To achieve maximum cleaning effect, cleaning cloths in most food-service establishments are usually soaked in dilute disinfectants whenever they are not in use. However, a majority of retail food service establishments fail to prepare correct dilutions of sanitizers and do not change them until they are deactivated resulting in bacterial contamination of the cloths (Tebbutt 1988). Such contaminated cloths are difficult to adequately disinfect after each use (Tebbutt 1991). Silver dihydrogen citrate sanitizing spray is an effective sanitizer which has broad spectrum and residual disinfectant characteristics against bacteria, fungi, and viruses. It damages an organism's DNA and protein structure and lysis its membrane resulting in the organism's death. Bacteria recognize citric acid as a food source and are attracted to it by chemotaxis, which enables SDC to enter the organism through membrane transport proteins (Pure Bioscience 2007). We investigated the efficacy of SDC sanitizing spray to reduce microbial counts on test surfaces after the surfaces had been cleaned with a cloth. The use of sanitizing sprays may contribute to the reduction of cross-contamination caused by inefficiently sanitized cleaning cloths. This study was conducted to: assess the effectiveness of selected cleaning cloths in cleaning food contact surfaces; determine the effectiveness of cloth-sanitizer combinations in reducing food contact surface contamination to acceptable levels; and determine if the detection capability of the two testing methods (ATP-B, and TPC) is the same.

As part of the comprehensive project aimed at studying cost effective treatments to minimize *Listeria monocytogenes* cross-contamination of ready-to-eat (RTE) meats by the in-store deli meat slicer, we investigated the effectiveness of moist heat, in combination with SDC sanitizer, to cause a significant reduction of *Listeria* strains inoculated on coupons cut from deli meat slicers. This study was intended to

further ensure the reduction of contamination on food contact surfaces, particularly the reduction of *L. monocytogenes* contamination on deli meat slicers after cleaning, disinfection and sanitization.

Characterization and taxonomy of the genus *Listeria*

The genus *Listeria* comprises a group of short, gram-positive, non-spore-forming, rod-shaped, and intracellular bacterial pathogens measuring 0.5 µm in width and 1 to 2 µm in length (Dongyou Liu 2006, Ryser and Donnelly 2001). Members of the genus are facultative organisms which, nevertheless, prefer a microaerophilic environment (Ryser and Donnelly 2001). The optimum growth temperature for *Listeria* species ranges between 30 and 37°C; however, these psychrotrophic foodborne pathogens can multiply at temperatures ranging between 1 to 45°C (Ryser and Donnelly 2001). It can grow at a water activity (a_w) of 0.90 and in solutions containing more than 10% NaCl (Ryser and Donnelly 2001). When grown on nutrient agar, the colonies, which measure 0.2 to 0.8 mm in diameter after 24 h of incubation, are characteristically smooth, bluish grey and slightly raised (Ryser and Donnelly 2001). When incubated at 25°C in tubed motility media, they exhibit a typically umbrella-shaped growth and a tumbling motility is observed in wet mounts (Ryser and Donnelly 2001). The optimum pH of *Listeria* is 7, but it can also grow in laboratory media at pH 4.4 to 9.6 (Ryser and Donnelly 2001). They grow rapidly in most commonly used bacteriological media and broth cultures typically become turbid within 8 to 24 h of incubation at 35°C (Ryser and Donnelly 2001). Their ability to survive and proliferate at refrigeration temperatures are some of the numerous factors that make the control of *Listeria* difficult.

Listeria species share many morphological and biochemical characteristics which have frequently been exploited for their differentiation from each other and from other bacteria (Fig. 1) as discussed below. They are catalase and methyl red positive, oxidase and indole negative. *Listeria monocytogenes* exhibits ß-hemolysis on blood agar, a characteristic believed to be related to pathogenicity. Other biochemical features shown by *Listeria* species include fermentation of carbohydrates to acid without gas, hydrolysis of esculin and sodium hippurate but not urea, production of ammonia from arginine and negative reactions for hydrogen sulfide production, nitrate reductase, gelatin liquefaction, and starch

hydrolysis. These characteristics are used for presumptive identification of *Listeria* species (Dongyou Liu 2006, Ryser and Donnelly 2001).

The eight currently recognized *Listeria* species include *L. monocytogenes*, *L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. marthii* and *L. rocourtiae*. Rocourt *et al.* (1992) presented evidence that *L. grayi* and *L. murrayi* should be considered as a single species called *L. grayi*. Recently two new *Listeria* species; *L. marthii* and *L. rocourtiae* have been described by Graves *et al.* (2010) and Leclercq *et al.* (2010), respectively. These species were respectively named after Emeritus Professor Elmer H. Marth, for his research and contributions on *L. monocytogenes* and Jocelyne Rocourt, a French bacteriologist, who contributed significantly on the taxonomy of the genus *Listeria*. *L. marthii* has not yet been linked to human or animal disease and *L. rocourtiae* has been reported to be avirulent (Graves *et al.* 2010, Leclercq *et al.* 2010). Only the hemolytic *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* have been linked with human pathogenicity (Cocolin 2002). *L. monocytogenes* is the only species of the genus *Listeria* that has been involved in known food-borne outbreaks of listeriosis (Cocolin 2002). *L. ivanovii* is mostly an animal pathogen that rarely occurs in man (Low and Donachie, 1997). Listeriosis associated infections caused by *L. ivanovii* and *L. seeligeri* have been reported to be extremely rare in humans (Cocolin 2002, Hitchins and Jinneman 2011). *Listeria* species cause morbidity and mortality in both humans and livestock. There are currently 13 strains of known *L. monocytogenes* serotypes including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). Although human listeriosis may be caused by all the serotypes, most of the strains isolated from human cases and foods belong to serotype 1/2a, 1/2b and 4b (Farber and Peterkin 1991).

L. monocytogenes has been identified using phenotypic methods by profiling the genetic products of the organism through quantification of biochemical antigenic and bacteriophage characteristics (Liu 2006). Phenotype-based methods have, however, been reported to potentially yield ambiguous results given the likelihood of variations with changing external conditions due to growth phase and spontaneous genetic mutations (Liu 2006). Recently developed precise molecular techniques have made it possible to

identify and differentiate *Listeria* species from each other and from other pathogens by targeting the organism's specific genes, with less interference from the stated confounding factors (Fig. 1) (Liu 2006).

Isolation and selective enrichment and plating

L. monocytogenes samples collected from food processing surfaces are detected by swabbing the surfaces with sterile sponges or gauzes moistened with neutralizing buffer (USDA/FSIS 2009). The swabbing material is then placed in University of Vermont Broth (UVM) or *Listeria* Repair Broth (LRB), stomached for 2 min to dislodge cells and then incubated at 30°C for 24 h (USDA/FSIS 2009). Following incubation, the primary enrichment culture is then streaked onto Modified Oxford Media (MOX) plates, which are incubated for 48hrs at 35 ± 2°C and then examined for *Listeria*-like colonies. Typical *Listeria* colonies are rounded 1-mm colonies surrounded by a black zone (Ryser and Donnelly 2001). Additionally, 10 µl of the UVM culture may be pipetted into 10 ml of Frazer's secondary enrichment broth (FB) and examined for blackening after 48 h of incubation at 35 ± 2°C (USDA/FSIS 2009). Blackened FB cultures are then streaked onto MOX plates which are incubated at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved on MOX plates (Ryser and Donnelly 2001, USDA/FSIS 2009).

Biochemical confirmation methods

Biochemical tests take advantage of the many morphological and biochemical characteristics of *Listeria* species mentioned above. *L. monocytogenes* will appear as slim short rods with slight rotating or tumbling motility on a heavy wet mount suspension in 0.85% saline as a suspending media. Non-*Listeria* cells will appear as cocci, large rods or rods with rapid motility. On a Gram stain reaction and catalase activity *Listeria* species are gram positive and catalase positive. *L. monocytogenes* and *L. seeligeri* will produce slight clearing zones around a heavily inoculated stab on a thickly poured and well-dried TSA media containing 5% sheep blood agar, whereas *L. innocua* will not produce the clearing zone at all and *L. ivanovii* will produce a strong hemolytic zone after incubation at 35°C for 48 h.

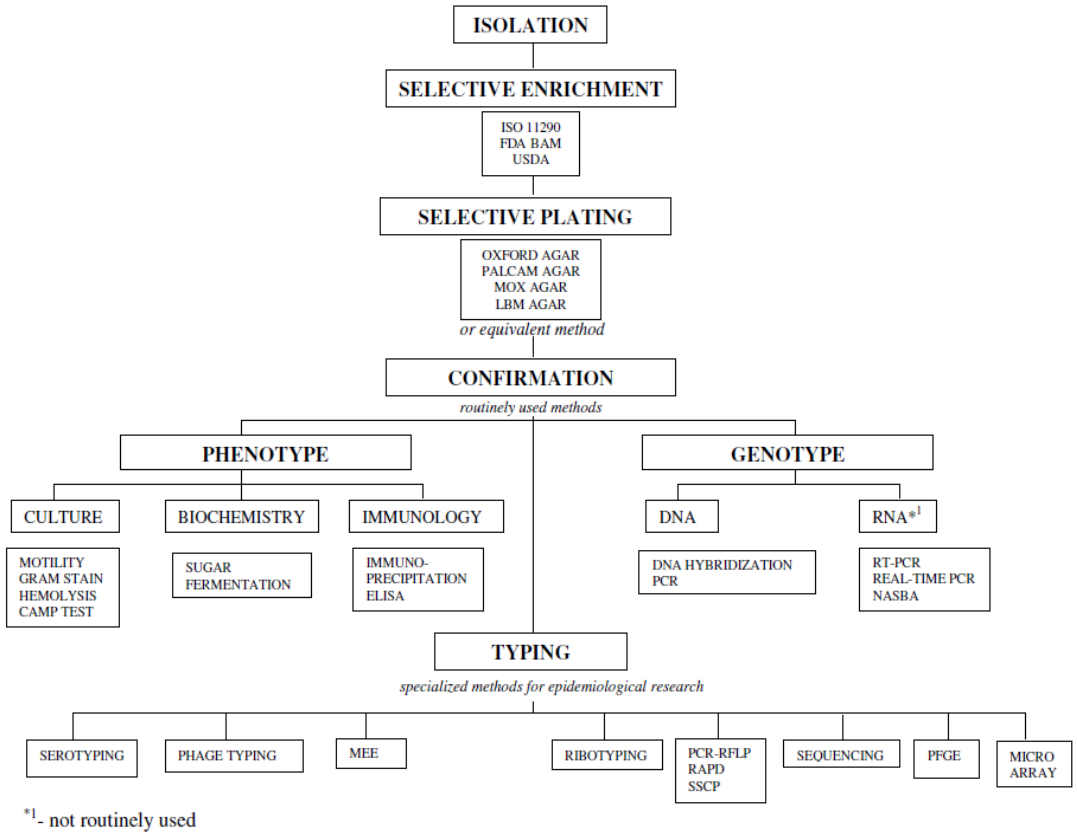

Fig. 1. Overview of isolation, identification and typing methods for Listeria and L. monocytogenes in foods and environmental samples. (Adopted from Gasanov *et al.* 2005)

After one week of incubation at 35°C on tubes of purple carbohydrate fermentation broth containing glucose bile esculin, maltose, rhamnose, mannitol, and xylose with or without Durham tubes, all *Listeria* species will produce acid from glucose, esculin, and maltose, whereas all *Listeria* species, except *L. grayi* will be negative for mannitol (Ryser and Donnelly 2001). When results from blood agar stabs are questionable the Christie-Atkins-Munch-Peterson (CAMP) test becomes useful in differentiating among *Listeria* species (Ryser and Donnelly 2001). The hemolytic activity of *L. monocytogenes* and *L. seeligeri* is stronger near the *Staphylococcus aureus* streak whereas *L. ivanovii* hemolysis is enhanced near the *Rhodococcus equi* streak. *L. innocua, L. welshimeri* and *L. grayi* will not react because they are not hemolytic in this test. This test will, therefore, differentiate *L. ivanovii* from L. seeligeri as well as the weakly hemolytic *L. seeligeri* from *L. welshimeri*. The CAMP test should be conducted for typical *L.*

monocytogenes isolates which do not exhibit hemolytic activity before being branded as non-hemolytic *L. innocua* (Ryser and Donnelly 2001, USDA/FSIS 2009).

Subtyping and molecular detection methods

Subtyping and molecular detection methods help to characterize *L. monocytogenes* isolates beyond species level particularly during foodborne disease outbreaks where the vehicle of infection needs to be confirmed and the source of contamination traced back to a particular food processing facility or environment (Ryser and Donnelly 2001). Different conventional and phenotypic subtyping as well as genetic subtyping methods for *L. monocytogenes* have, therefore, been used to discriminate among strains or subtypes (Liu 2006, Ryser and Donnelly 2001). Subtyping methods, such as serotyping, are based on the fact that different strains of bacteria differ in the antigens they carry on their surfaces (Wiedmann 2002). These surface antigens can be detected by antibodies and antisera (Liu 2006). However, serotyping does not have good discriminatory power compared with other subtyping methods especially molecular subtyping methods (Liu 2006). Phage typing, for example, characterizes bacterial isolates by their susceptibility to lysis by a standard set of phages and requires a standardized reference phage set for comparison among laboratories (Wiedmann 2002). It has an overall percent typability of *L. monocytogenes* strains ranging between 52 and 78% with a typability of more than 90% for the serotype ½ strains (Farber and Peterkin 1991). Isoenzyme typing has been found to be useful in either confirming or rejecting a common source as the cause of an outbreak of food-borne listeriosis (Farber and Peterkin 1991). Multilocus enzyme electrophoresis (MEE) differentiates bacterial strains by variations in the mobility of the different constituent enzymes in the electrophoretic gel. This method usually provides 100% typability, but its standardization between laboratories has proven to be difficult (Wiedmann 2002). Moreover, MEE appears to be less discriminatory than some DNA-based subtyping methods (Wiedmann 2002). Another typing method is monocine typing whose usefulness; however, is limited by the fact that only 56 and 59% of serovars 4b and 1/2a, respectively, have been found to be producers of monocines (Farber and Peterkin 1991). Molecular subtyping or DNA-based subtyping methods have markedly improved the ability to differentiate strains and subtypes. DNA-based subtyping methods include DNA

fingerprinting using both restriction enzyme analysis (REA) and ribotyping, the latter having been found to be more discriminatory than either serotyping or phage typing, but equivalent to isoenzyme typing despite being less discriminating than REA in some case (Farber and Peterkin 1991). The Pulsed-Field Gel Electrophoresis (PFGE) technology, a molecular subtyping method being used in reference laboratories in the USA, enables molecular subtyping or fingerprinting of pathogens isolated from both humans and foods. PFGE yields a high level of sensitivity for discrimination of *L. monocytogenes* strains, and is often designated the current gold standard for discriminatory ability (Liu 2006, Wiedmann 2002). DNA sequencing-based subtyping methods, such as Multilocus sequence typing (MLST), are also being used to differentiate bacterial subtypes and to determine the genetic similarity of isolates. MLST is a molecular subtyping approach that uses DNA sequencing of multiple genes or gene fragments, usually of numerous housekeeping genes or virulence genes (Liu 2006). The subtyping of pathogens has become essential in helping public health agencies to identify groups of related illnesses and then link specific food products to specific human illnesses when the patterns match (USDA/FSIS 2003a). Methods for isolating and identifying *Listeria* have been reported to have a 10 to 15% false-negative rate (Montville and Matthews 2008). This shortcoming has raised the argument that it may not be fair to demand "zero tolerance" for *Listeria* in food when there is no 100% reliable test (Montville and Matthews 2008). This policy is discussed further in a section below.

Persistence of *L. monocytogenes* in the environment

The resilient properties of *Listeria* species allow the organism to be present in a variety of environments, including soil, mud, water, silage, decaying vegetation, effluents, feces and foods. Its ubiquitous nature has been further recognized by its association with various animals such as mammals, birds, fish, amphibians, and insects (Ryser and Donnelly 2001, Ramaswamy *et al.* 2007). The primary habitat of *L. monocytogenes* is believed to be soil and vegetation where it leads a saprophytic life, and its presence in such environments makes it possible for wild and domestic animals to contract and transmit the organism (Ryser and Donnelly 2001). *L. monocytogenes* has been isolated in samples taken from grazing areas, stale water supplies and contaminated animal feed (Ryser and Donnelly 2001). Both

animals and humans can be asymptomatic carriers of *Listeria* (Ryser and Donnelly 2001). It has been reported that more than 5% of the general population is assumed to be asymptomatic carriers (Ryser and Donnelly 2001, Ramaswamy *et al.* 2007). Moreover, *Listeria* species are persistent even in extreme pH, temperature and salt conditions. Exposure of *Listeria* to mild acidic pH of 5.5 prompts the development of acid tolerance mechanisms in the cells, resulting in their resistance to severe acidic conditions (O'Driscoll *et al.* 1996). At low temperatures (1 to 4°C) *Listeria* also develops mechanisms that enhance its survival. Gandhi and Chikindas (2007) reported four of these mechanisms as including changes in membrane composition, changes in gene expression and induction of proteins, the ability to accumulate compatible cryoprotectant solutes, and the role of general stress sigma factor (σ^B). However, Doyle *et al.* (2001) have reported that *L. monocytogenes*'s resistance to heat is influenced by such factors as stress conditions (including exposure to sub-lethal heat and acidity among others), previous growth conditions, composition of the heating conditions and strain variation. Crandall *et al.* (2010) have demonstrated that the organism can survive dry heat at 82°C for 15h. It has also been reported that this organism can survive sodium chloride concentrations of up to 10% (Ryser and Donnelly 2001). The ubiquitous nature of the organism and its ability to survive and proliferate in the environment and host species, combined with the number of possible transmission routes, complicates its control in the environment.

***L. monocytogenes* in food**

The ability of *L. monocytogenes* to tolerate and to survive for very long periods in harsh conditions further allows for its widespread nature in a variety of raw and processed foods. Rocourt and Cossart (1997) reported several food items that have been associated with *Listeria* contamination or implicated in a number of human listeriosis outbreaks including milk and dairy products, seafood and fish products, beef, pork, fermented sausages, radishes and cabbage. Several studies have revealed that contamination of foods by *L. monocytogenes* can occur at any point in the food chain, including farms, in food processing plants, in retail establishments and in the home (Lappi 2004, Nightingale 2005, Saunders 2006). This is evidenced by its detection in a wide range of foods as described above. It has been shown that the ability of *L. monocytogenes* to survive and proliferate on meat is dependent on temperature, pH of

the meat, type of tissue, and initial miroflora already present on the meat's surface (Farber and Peterkin, 1991). Petran and Zottola (1989) have noted that despite the fact that *Listeria* can be easily inactivated by cooking and pasteurization, exposure of food in the processing plant environment after cooking but before packaging may result in cross-contamination. For this reason *L. monocytogenes* remains an important problem in ready-to-eat foods such as delicatessen meats (Tompkin, 2002). It has been demonstrated that *L. monocytogenes* has the ability to colonize, proliferate, and thrive in the food processing environment, including food processing equipment, for long periods (Lappi *et al.*, 2004, Kabuki *et al*., 2004). *Listeria* can easily adapt in the environment and can even survive freezing, surface dehydration and spray chilling conditions, but it can be easily killed with proper cooking (Seeliger and Jones 1986, Junttila *et al.*1988).

L. monocytogenes transmission routes

L. monocytogenes has been reported to cause infection by several transmission routes including consumption of contaminated foods (Schlech *et al.*1983, Fleming *et al.*1985, Linnan *et al.* 1988), mother to fetus in utero transmission, direct infection of the fetus at the time of birth, or by direct contact with the organism, which causes lesions on the skin (McLauchlin 1990). Most *L. monocytogenes* infections are, however, acquired through ingestion of contaminated food (Montville and Matthews 2008). An infected pregnant mother transmits the organism to her unborn child resulting in in utero infection of the fetus which may eventually lead to premature birth, neonatal illness and death (Nightingale *et al*. 2004). In some rare cases infected veterinarians, farmers, and abattoir personnel handling contaminated meat have been proof that the organism can also be transmitted directly from infected animals to humans (Nightingale *et al*. 2004). People who have contracted listeriosis from animals characteristically exhibit localized skin infections rather than the systemic syndrome (Nightingale *et al.* 2004).

Characteristics of listeriosis

Unlike most pathogens, which produce toxins or proliferate in the blood system, *L. monocytogenes* spreads through cell-to-cell transmission. The pathogen enters the host's cells, proliferates inside the cells and passes directly into adjacent cells (Montville and Matthews 2008). This mode of transmission in the host limits the effectiveness of both antibiotics and circulating antibiotics because of

the resultant reduced exposure of the pathogens (Montville and Matthews 2008). Its ability to pass through cell membranes makes it possible for the pathogen to penetrate into the brain and placenta (Montville and Matthews 2008). *L. monocytogenes* causes clinical syndromes categorized as febrile gastroenteritis, perinatal infection, and systemic infections marked by central nervous system infections with or without the presence of bacteria in blood (Drevets and Bronze 2008). The organism is also reported to go through three stages before it can cause listeriosis, which include ingestion of the organism; penetration of the gastrointestinal lining and phagocytosis of macrophages; and finally lysis of the macrophages resulting to septicemia and the invasion of target organs such as the central nervous system and the placenta (Ryser and Donnelly 2001). Listeriosis is a severe illness common in high risk groups who have pre-disposing conditions that suppress their immune system, including the elderly, pregnant women, newborns and immuno-compromised individuals, such as cancer, organ transplant, human immunodeficiency virus, and immunosuppressive therapy patients (Montville and Matthews 2008). Predisposing conditions often associated with listeriosis include, among others, neoplastic disease, immunosuppression, pregnancy, extremes of age, diabetes mellitus, alcoholism, cardiovascular disease, renal collagen diseases and hemodialysis failure (Farber and Peterkin 1991). Some cases are, however, occasionally found in non-pregnant adults where *L. monocytogenes* causes septicemia, meningitis, and meningoencephalitis (Montville and Matthews 2008, Drevets and Bronze 2008). Febrile gastroenteritis incubation period is estimated to be 6 to 49 h, and most individuals suffering from the gastroenteritis form of listeriosis exhibit mild gastrointestinal symptoms, commonly seen in healthy individuals, such as diarrhea, fever, abdominal pain, chills, headache and myalgia (Drevets and Bronze 2008). Febrile gastroenteritis is a mild self-limiting disease which has been observed in normal hosts and whose symptoms such as fever and diarrhea may take average durations of 27 and 42 h, respectively (Drevets and Bronze 2008). Most gastroenteritis patients recover without antimicrobial treatment (Drevets and Bronze 2008). The invasive form of listeriosis is most often observed in certain well-defined high-risk groups as previously described. However, it has also been occasionally seen in patients with no

predisposing conditions (Ryser and Donnelly 2001). The increase in the number of the aging population combined with an increase in the use of immunosuppressive medications and the continuing epidemic of the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV and AIDS) have increased the number of people at risk of listeriosis infection (Ryser and Donnelly 2001). Forms of the invasive listeriosis include maternal-fetal/neonatal listeriosis (perinatal infection) and bacteremia (systemic infection) with or without cerebral infections such as meningitis, meningoencephalitis, rhombencephalitis or brain abscess (Ryser and Donnelly 2001, Drevets and Bronze 2008). Fever, malaise, fatigue, and abdominal pain are experienced by bacteremia patients, whereas for patients in which listeriosis has progressed to the central nervous system, fever, malaise, seizures, ataxia and altered mental status are observed (Ryser and Donnelly 2001).

Infectious dose - the dilemma over tolerance levels

The infectious dose of the organism is not known; however, Montville and Matthews (2008) and Lianou and Sofos (2007) reported that the infectious dose is dependent on the susceptibility status of the host, the virulence of the strain, and the type of food product involved. Relatively lower doses may, therefore, still cause infection in high-risk populations. Analyses of listeriosis cases indicate that there are usually >100 CFU of *L. monocytogenes* per gram in foods linked to outbreaks, even though lower levels (<0.3 CFU/g) have been observed in some cases such as the frankfurters incriminated in the 1998 listeriosis outbreak (Montville and Matthews 2008). In this outbreak the serotype 1/2a strain was detected in meat samples at levels as high as 3000 CFU/g, whereas the serotype 4b isolates were present in quantities of <0.3 CFU/g (Mead *et al.* 2006). The uncertainty with *L. monocytogenes* infectious dose combined with its pathogenicity has caused considerable controversy over the years with regards to the regulation of the tolerance levels of *L. monocytogenes*. Since 1989 the United States (FSIS) has been implementing a “zero tolerance” policy against *L. monocytogenes* in 25g samples of ready-to-eat products and has been conducting a monitoring program within plants to test for *L. monocytogenes* (FSIS/USDA 1999). The argument is that there is no need for a “zero tolerance” in low-risk foods because they do not support the growth of *L. monocytogenes* to high numbers if they are not held for long periods at

temperatures that permit growth. Moreover, it is also argued that if the probability of illness from low-risk food is 1 in 100,000 for at risk populations, then the tolerance of < 100 CFU/g should be accepted. It is also argued that the "zero tolerance" regulation does not offer any benefit as the approximately 0.7 per 100,000 listeriosis incidence is similar to that in European countries where a <100 CFU/g tolerance level is used. Critics view this as a barrier to trade. This is evidence that more research is still required in this area (Montville and Matthews 2008). It is worth noting, however, that there is no epidemiological evidence that demonstrates whether a zero or non-zero tolerance policy leads to a greater rate of listeriosis. The reported annual incidence of human listeriosis ranges from 0.2 to 11.3 cases per million persons, 0.3 to 7.5 cases per million persons in Europe, 4.4 cases per million persons per year in the United States, and 3 cases per million per year in Australia. However, the accuracy of these values is dependent on the effectiveness of the individual countries' national listeriosis surveillance systems (WHO/FAO 2004).

Sporadic listeriosis cases

Listeriosis was first recognized as an animal disease and, prior to 1940, *L. monocytogenes* was already recognized as pathogenic to both animals and humans. Despite its first definitive description by Murray *et al*. (1926) and the reported listeriosis cases in the subsequent decades, it was its recognition as a foodborne human pathogen in the 1980s that quickly sparked interest among food manufacturers and government bodies to learn more about the pathogen (Low and Donache 1997, Ryser and Donnelly 2001, Drevets and Bronze 2008). The first confirmed cases of listeriosis, after the initial description of *L. monocytogenes,* were isolated from sheep in 1929 by Gill and by Nyfeldt from humans (Farber and Peterkin 1991). Sporadic listeriosis cases have been reported since then and these cases were mainly detected in workers in contact with infected animals (Farber and Peterkin 1991). Sporadic listeriosis is a rare disease, whose incidence is estimated at 7.4 cases per million people in the United States (Montville and Matthews 2008). However, most human listeriosis cases are sporadic and may be unrecognized sources of outbreaks (Montville and Matthews 2008). Exposure to *L. monocytogenes* is common, but listeriosis is rare, which may be due to a number of reasons including high human resistance or the low

pathogenicity of most strains (Montville and Matthews 2008). Listeriosis outbreaks receive considerable attention because of the severity of the disease (Montville and Matthews 2008). The worldwide incidence of neonatal cases of invasive listeriosis ranges from 0.1 to 1.1 cases per 105 people and is reported to be present in 47%, on average, of infected patients with an average case-fatality rate of 36% (Siegman-Igra *et al.* 2002).

Foodborne listeriosis outbreaks

The first confirmed outbreak of human foodborne listeriosis recorded in literature occurred in Canada, Nova Scotia, in 1981 and it was traced to consumption of *Listeria*-contaminated coleslaw. Of the 41 listeriosis cases in this outbreak, 7 were non-pregnant adults and 34 mother-infant pairs of which 15 infants (44%) and 2 adults died over a 6-month period, giving an overall mortality rate of 41%. The coleslaw was reported to have been prepared from cabbages grown in fields fertilized with both raw and composted manure from sheep, two of which had died from listeriosis. Serotype 4b was involved in this outbreak where the cabbage was reported to have been held in cold storage for a long time thus providing suitable environment and enough time for the pathogen to grow (Ryser and Donnelly 2001, Montville and Matthews 2008). This outbreak was followed by more outbreaks related to foods such as dairy, beef, poultry, and fish products (Montville and Matthews 2008) some of which are discussed below. Another outbreak with a mortality rate of 29% was experienced in 1983 where 49 listeriosis cases were reported over the duration of the outbreak, of which 42 were reported to be immune-compromised adults and 7 were mother-infant pairs. This outbreak was linked to pasteurized whole milk and 2% milk; however, *L. monocytogenes* was never isolated from the implicated milk, but from 15 samples of raw milk from the factory and several farms and milk cooperatives supplying the factory. This raised doubts as to the involvement of milk in this outbreak. The inefficiency of typing methods could also be a confounding factor in this case. Moreover, the epidemic strain 4b was not among the strains that were isolated. Contaminated Jalisco brand Mexican-style cheese was reported to be responsible for almost 300 listeriosis cases in a 1985 outbreak in California over an 8-month period. There were 142 cases reported by the Los Angeles County, 93 of which were pregnant women or their infants and 49 were non-pregnant

immune-compromised adults. Almost one-third (48) of the patients died, giving a mortality rate of 33.8%. Approximately 10,000 people were reported to have consumed the cheese suggesting that the overall attach rate was very law. Inadequate pasteurization of milk and mixing of raw milk with pasteurized milk was implicated in this outbreak. Further investigation revealed that there was a widespread occurrence of *L. monocytogenes* in the cheese plant environment which, on contamination of the milk, it managed to grow at the 6.6 pH of the final product. Phage typing and other DNA fingerprinting methods revealed that the patient and cheese isolates of *L. monocytogenes* serotype 4b were identical, thus confirming the implication of cheese in this outbreak (Ryser and Donnelly 2001, Montville and Matthews 2008). On another note, in 1994, 54 out of 60 (90%) previously healthy people presented with gastroenteritis listeriosis symptoms 9 to 32 h after consuming pasteurized chocolate milk at a picnic in Elizabeth, Illinois. Symptoms included diarrhea, fever, chills, nausea and vomiting. Twelve more cases were reported in Illinois, Wisconsin and Michigan. This time the incriminated serotype in this outbreak was *L. monocytogenes* serotype 1/2b which was isolated from unopened containers of chocolate milk at levels of 10^8 to 10^9 CFU/ ml. The chocolate milk taste and quality were reported to be poor. Only four people required short hospitalization with one woman delivering a healthy baby 5 days after experiencing a 6-h bout of diarrhea. Dalton *et al.* (1997) concluded that *L. monocytogenes* causes gastroenteritis with fever, and that sporadic cases of invasive listeriosis may be due to unrecognized outbreaks caused by contaminated food. Between August 1998 and February 1999, 101 cases of listeriosis were reported in 22 states in the United States. This outbreak was associated with the consumption of hot dogs. Over 80% of the patients were adults, of which >60% had predisposing conditions and the mortality rate was estimated at 21% which included 15 deaths and 6 miscarriages. The implicated *L. monocytogenes* strain was serotype 4b. Further analysis revealed the organism belonged to pulse-field gel electrophoresis type E which was not common in the USA and in other countries. The beginning of the outbreak coincided with a major renovation which involved the removal of a large ventilation unit near the hot dog packaging line (Ryser and Donnelly 2001). In 2000, a 7-month listeriosis outbreak, involving deli turkey meat, was reported in 10 states in the USA. *L. monocytogenes* serotype 1/2a was implicated in this outbreak and the

pulse-field gel electrophoresis revealed identical patterns. Twenty-nine individuals were infected in this outbreak, 8 of which were perinatal and 21 non-perinatal cases. The median age for the non-perinatal cases was 65 years (ranging between 29 to 92 years), 62% of which were females. The outbreak resulted in four deaths and three stillbirths. Recently, in 2011, a total of 146 persons infected with any of *L. monocytogenes* were reported to CDC from 28 states, between July 31 and December 8, 2011. Thirty deaths were reported including one miscarriage by a woman who was pregnant at the time of illness. This outbreak was traced to contaminated cantaloupes linked to Jensen Farms in Holly, Colorado. Serotypes 1/2a and 1/2b were implicated in the current outbreak (Idaho Department of Health and Welfare 2011).

Policies and regulations relating to *L. monocytogenes*

Listeriosis outbreaks have continued to occur despite stringent measures that have been put in place in the USA. The USA Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 342(a) (1) definition of food adulteration encompasses, among other things, food contaminated with pathogenic organisms. The Federal Meat Inspection Act (FMIA; 21 U.S.C. 601 et seq.) and the Poultry Products Inspection Act (PPIA; 21 U.S.C. 451 et seq.), administered by the FSIS, also ensure that meat, poultry, and egg products prepared for distribution in the market are wholesome, not adulterated, and properly marked, labeled, and packaged. The FMIA and PPIA prohibit anyone from selling, transporting, offering for sale or transportation, or receiving for transportation in the market, any adulterated or misbranded meat or poultry product (21 U.S.C. 610, 458). Following the late 1980's outbreaks, it was evident that *L. monocytogenes* presented a serious problem in deli meats and other processed products. The United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) worked with food processing plants to improve their procedures and established a "zero tolerance" policy for the pathogen in ready-to-eat products. Since 1989, the USDA-FSIS has been implementing the "zero tolerance" policy for *L. monocytogenes* in ready-to-eat products such as hot dogs and luncheon meats and continue to carry out a monitoring program within food processing plants to test for the pathogen (USDA-FSIS 1999). The "zero tolerance" policy means that the detection of any *L. monocytogenes* in either of two 25-gram samples of ready-to-eat food renders the food adulterated as defined by the Federal Food, Drug, and

Cosmetic Act, 21 U.S.C. 342(a)(1) and the FMIA or PPIA (21 USC 453(g) or 601(m)) . By 1999 no significant changes had been observed in listeriosis rates after its 44% decline between 1989 and 1993 (USDA-FSIS 1999). During this time the FSIS sampling program was analyzing eight categories of beef products for *L. monocytogenes* including: beef jerky; roast beef, cooked beef, and cooked corned beef; sliced ham and luncheon meat; small-diameter sausage; large-diameter sausage; cooked, uncured poultry; salads and spreads, and; dry and semi-dry fermented sausage (USDA-FSIS 1999). Of the 3,547 samples of ready-to-eat products analyzed in 1998, 2.5% (90) tested positive for *L. monocytogenes*. Hot dog samples alone were 4.4% positive for *L. monocytogenes* over a period of 3 years between 1993 and 1996 (USDA-FSIS 1999). The FSIS sampling program requires food processing plant management to voluntarily put the product, being investigated, on hold until the results are out. If a positive sample is discovered and the implicated product has been distributed to customers, FSIS requires that the plant initiates a voluntary product recall (USDA-FSIS 1999). An inspector may also, at any time during his/her routine investigation, take a sample for analysis, apart from the monitoring program, and then carry out follow-up testing in response to a positive sample (USDA-FSIS 1999).

In 1990 the Department of Health and Human Services (DHHS) launched the Healthy People 2000 of which Food and Drug Safety was one of the 22 priority areas which focused, among other things, on reducing foodborne illnesses (FDA-CFSAN/USDA-FSIS/CDC 2003). Healthy People established the objective to reduce infections caused by *Salmonella, Campylobacter jejuni, E.coli* O157:H7 and *L. monocytogenes*, because of the frequency and severity of these food-borne infections. By the end of 2000 *L. monocytogenes* cases had been reduced by 29% (FDA-CFSAN/USDA-FSIS/CDC 2003). The incidence of *L. monocytogenes* infection decreased from 0.5 to 0.3 cases per 100,000 people per year between 1996 and 2001, but it plateaued between 2001 and 2002 as shown in the graphs below (Fig. 2). The Healthy People 2010 goals for national health promotion and disease prevention called on federal food safety agencies to reduce foodborne listeriosis by 50% by the end of the year 2005 (FDA-CFSAN/USDA-FSIS/CDC 2003). By 2007, the estimated percent of *L. monocytogenes* deaths remained the second highest at 27.6% to salmonella species at 30.6% (Sondik 2007). Between 1997 and 2002 *L.*

monocytogenes infections declined from 0.47 cases per 100,000 people to 0.26 cases per 100,000 population, achieving 91 percent of the targeted change (FDA/USDA-FSIS 2010).

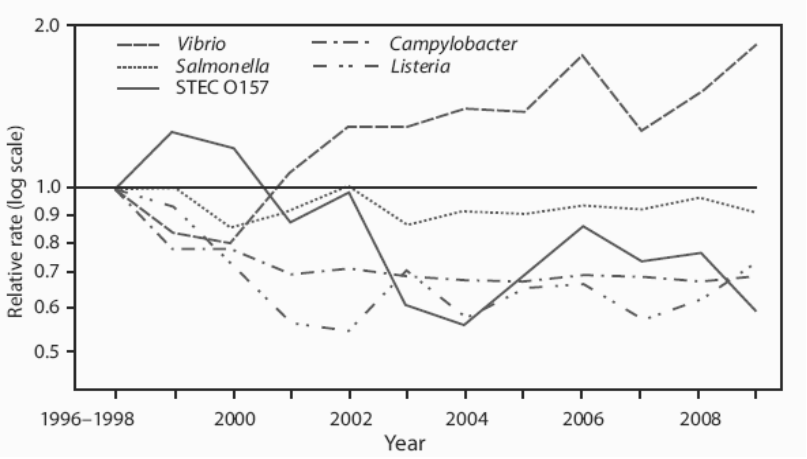

FIG. 2 Relative rates of laboratory-confirmed infections with *Campylobacter*, *Shiga* toxin- producing *Escherichia coli* (STEC) O157, *Listeria*, *Salmonella*, and *Vibrio* compared with 1996-1998 rates, by year. Foodborne Diseases Active Surveillance Network (FoodNet), United States, 1996-2009 (Adopted from CDC 2010)

The Healthy People 2010 target was 0.24 *L. monocytogenes* cases per 100,000 people; however, the incidence rate remained at 0.3 cases per 100,000 people by 2009 (FDA/USDA-FSIS 2010). To further achieve an incidence rate of 0.25 *L. monocytogenes* cases per 100,000 people by the end of 2010, additional targeted measures were needed. The DHHS, FDA's Center for Food Safety and Applied Nutrition (FDA-CFSAN) conducted a risk assessment in collaboration with the U.S.DA's Food Safety and Inspection Service (USDA-FSIS) and in consultation with the DHHS Centers for Disease Control and Prevention (CDC). The purpose of the assessment was to systematically examine the available scientific data and information to estimate the relative risks of serious illness and death associated with the consumption of different types of ready-to-eat (RTE) foods that may be contaminated with *L. monocytogenes*. The risk assessment studied current science and was intended to use the models developed from it as one of the tools that food safety regulatory agencies will consider when evaluating

the effectiveness of current and future policies, programs, and regulatory practices to reduce the public health impact of *L. monocytogenes*. *L. monocytogenes* had the highest case fatality rate (21%) and the highest hospitalization rate (90.5%) among all the food pathogens investigated by the CDC in 2000. The risk assessment, therefore, focused on the severe public health consequences (FDA-CFSAN/USDA-FSIS/CDC 2003). The *L. monocytogenes* risk assessment was one of the initiatives of the Healthy People 2010 aimed at evaluating achievements of the goals for national health promotion and disease prevention. This national health promotion program involved federal, state, and local government agencies, voluntary and nonprofit organizations, communities, and individuals together to lead in a fight to improve the health of the American people. It brought together the FSIS, CDC, state and local health departments with the goal of determining the sources of *L. monocytogenes* (USDA/FSIS 1999). The Healthy People 2010 also brought together state and federal agencies to review regulatory, enforcement, and educational strategies for reducing illness associated with the pathogen (FDA-CFSAN/USDA-FSIS/CDC 2003).

The Final Rule (9 CFR Part 430) established in 2003, set out requirements for establishments that produce RTE meat and poultry products, which are exposed to the environment after lethality treatments and that support the growth of *L. monocytogenes*, to test food contact surfaces for *Listeria* species to verify that they were controlling the presence of *L. monocytogenes* within their processing environments. It required such establishments to have, in their hazard analysis and critical control point (HACCP) plans, or in their sanitation standard operating procedures or other prerequisite programs, controls that prevent product adulteration by *L. monocytogenes*. Under the final rule, food establishments that have HACCP programs that prevent product adulteration by *L. monocytogenes* are exempt from testing requirements because the HACCP regulations require on-going monitoring and verification to demonstrate that the food safety system is working. Moreover, establishments are required to report to FSIS data and information pertaining to their *L. monocytogenes* controls as well as information on the amount of produced products affected by the regulations. The regulations permit the food-processing establishments to make claims on the labels of their RTE products regarding the processes they use to eliminate or reduce

L. monocytogenes or suppress or limit its growth in the products (USDA-FSIS 2003a, Gallagher *et al.* 2003).

Economic burden of listeriosis in the USA

A recent study by Scallan *et al.* (2011) estimated the annual number of foodborne listeriosis cases to be 1,591 in the United States alone. They further estimated that annual foodborne listeriosis related deaths comprised 19% of the 1,351 foodborne illnesses caused by 31 major foodborne pathogens in the USA. Scharff (2010) estimated the total cost per listeriosis case to be $1,695,143. He further estimated the total cost of foodborne listeriosis to be $8,823 million based on the report by Mead *et al.* (1999) which estimated foodborne listeriosis cases at 5,205.

Potential factors contributing to the emergence of listeriosis

The emergence of listeriosis has been related to improvements in hygiene resulting in the elimination of competing bacteria such as spoilage bacteria that prevent listeria from growing (Montville and Matthews 2008). The survival of *L. monocytogenes* has been reported to be reduced in competition with other bacteria, but its resilient properties allow it to thrive when competing bacteria are eliminated. *L. monocytogenes* is an opportunistic pathogen that attacks immunocompromized individuals. Listeriosis has, therefore, found an opportunity to infect more people as more people at risk increase due to the growing aging population, persistence of AIDS epidemic, use of immune-suppressants, organ transplants and many more such conditions (Montville and Matthews 2008). Good hygiene practices have become more challenging as small food outlets are increasingly being consolidated into large centralized facilities (Montville and Matthews 2008). The use of refrigeration of food for preservation has increased over the years. This has made it possible for *L. monocytogenes* to thrive in such freezing conditions where other foodborne pathogens do not grow (Montville and Matthews 2008). Moreover, there is growing demand for “fresh”, “minimally processed”, and natural foods that need little cooking or preparation and foods that do not contain preservatives which would otherwise prevent the growth of *L. monocytogenes* (Montville and Matthews 2008). Recent technological developments such as computerized databases,

DNA fingerprinting and the internet have made it possible to detect pathogenic strains that could not be detected in the past (Montville and Matthews 2008).

L. monocytogenes in the food-processing environment

Montville and Matthews (2008) reported that *L. monocytogenes* could enter food processing plants through soil on workers' shoes, clothing, vehicles, contaminated vegetables and animal tissue as well as through human carriers. They added that the food processing environment provides suitable conditions for the growth of *L. monocytogenes*. Such conditions include high humidity and nutrient levels. *L. monocytogenes* has been identified in moist areas such as floor drains stagnant water, floors, and processing equipment. It has also been reported to attach on surfaces such as stainless steel, glass and rubber. Bacteria enter foods as a result of contact with contaminated surfaces (Beresford 2001). Moreover, *L. monocytogenes* has the ability to form biofilms on food contact surfaces rendering them difficult to clean because cells in a biofilm can be significantly more resistant to disinfectants and sanitizers than planktonic cells (Ronner and Wong 1993). Lunden *et al.* (2000) demonstrated that persistent *L. monocytogenes* strongly attached to stainless steel surfaces within a short time (1 and 2 h), which could explain their survival in food processing plants. Taormina and Beuchat (2002) reported that *L. monocytogenes* can survive alkaline cleaning solutions commonly used for food processing equipment and that the subsequent use of sanitizers could significantly reduce *L. monocytogenes* from food contact surfaces. Heat processes have been shown to eliminate *L. monocytogenes* from food and food processing equipment and *L. monocytogenes* gets into processed foods predominantly by post-processing contamination. The organism often colonizes raw materials used in the food preparation establishments making it easy for it to recontaminate previously clean environments. Tompkin (2002) identified places in which *L. monocytogenes* has been detected within the food processing environment. These include hollow rollers on conveyors, cracked tubular support rods on equipment, the space between close-fitting metal-to-metal or metal-to-plastic parts, worn or cracked rubber seals around doors, on-off valves and switches for equipment, and saturated insulation. There is also some evidence that construction in an area adjacent to exposed RTE products can increase the risk of product contamination, probably due to dust that may be

dispersed throughout the area (Tompkin 2002). This may lead to the introduction of a new virulent *L. monocytogenes* strain in the food processing environment, which may increase the potential of product contamination if the organism establishes itself in a new niche in the environment in which RTE products are processed (Tompkin 2002). Examples of sources of contamination by *Listeria* species or *Listeria*-like organisms in RTE-food-processing operations include slicers, dicers, packaging machines and conveyors, among others (Tompkin 2002).

***L. monocytogenes* cross-contamination through deli meat slicers**

Over four decades ago, Gilbert and Maurer (1968) identified the risk of cross-contamination from one meat product to another as different products are sliced with the same deli meat slicer. Of the 362 people, who contracted typhoid in a 1964 Aberdeen outbreak, and known to have purchased food from a cold-meat counter in a supermarket, 131 had eaten corned beef and 138 had eaten unspecified cold meats, whereas 93 had eaten cold meats but not corned beef. All the meat had been cut using the same deli meat slicer. This was proof that a contaminated product may continually recontaminate the slicing machine, thus spreading the pathogen to other products.

The FDA-CFSAN/USDA-FSIS/CDC (2003) quantitative assessment of relative risk to public health from foodborne *L. monocytogenes* among selected categories of ready-to-eat foods ranked deli meat as the highest-risk ready-to-eat food vehicle of *L. monocytogenes* in the U.S. Similar findings were also reported in several studies (Lin *et al.*. 2006, Vorst *et al.* 2006). Lin *et al.* (2006) concluded that since *L. monocytogenes* can adhere to most materials including food processing equipment used in deli meat plants, slicing equipment, therefore, pose a potential vehicle of cross-contamination by *L. monocytogenes* during meat processing. Their study demonstrated that there was sufficient opportunity for transfer of *Listeria* in delicatessens via mechanical slicers, with the highest risk of consumer exposure coming from the first 10 slices produced on a contaminated slicer, after which intermittent transfer occurred up to 28 to 30 slices. They concluded that *L. monocytogenes* could be transferred from a commercial slicer and related equipment onto deli meat, and that the amount of *L. monocytogenes* transferred was associated with the number of cells inoculated onto the slicer blade. The transfer of *L. monocytogenes*, from food

contact surfaces, such as mechanical deli meat slicers and knife blades, to food products (or vice versa) has been extensively studied (Vorst *et al.* 2006, Keskinen *et al.* 2008, Sheen and Hwang 2008, Sheen 2008).

Several major listeriosis outbreaks in the United States have been traced to the consumption of delicatessen-sliced meat (CDC 2000, Gottlieb *et al.* 2006). Endrikat *et al.* (2010) reported that 83% of listeriosis deaths linked with deli meats is attributed to retail-sliced products, and that of these estimated deaths 69.8% occurred from retail-sliced products that did not possess a microbial growth inhibitor such as sodium diacetate and potassium lactate (Lin *et al.* 2006).

Of the 500 estimated annual listeriosis fatalities, 242 are believed to be traceable to delicatessen meats. It is estimated that 75% of all luncheon meats sold are being sliced at delicatessens, thus exposing more people to *L. monocytogenes* infection (Vorst *et al.* 2006). Gombas *et al.* (2003) reported a prevalence of 2.7% of *L. monocytogenes* from retail-sliced products compared with 0.4% from prepackaged commercially manufactured and sliced RTE products. Draughon *et al.* (2006) found a similar trend of 1.4% and 0.2% prevalence on retail-sliced and prepackaged products, respectively. Moreover, one study found that 60% of sampled retail food facilities were contaminated with *L. monocytogenes* (Sauders *et al.* 2006). Unlike food processing and manufacturing facilities, which usually deal with one or a few products, retail food establishments handle various types of RTE products which in most cases are open to the public for display and may require slicing (Tompkin 2002). This information provides clear evidence that serious attention needs to be given to the retail deli environment. Efforts aimed at minimizing contamination at delicatessens will, therefore, undoubtedly effectively reduce the incidence of listeriosis.

Strategies to control *L. monocytogenes* in the delicatessen environment

Tompkin (2002) has recommended, among other control strategies, the implementation of measures aimed at preventing the establishment and proliferation of *Listeria* in harborages or other sites that can lead to the contamination of RTE foods. The USDA-FSIS Final Rule (2003c) provides alternatives for controlling *L. monocytogenes* in post-lethality exposed ready-to-eat products from which

food processing establishments should choose to implement. These alternatives include: post-package pasteurization, which requires establishments to employ both a post-lethality treatment and a microbial growth inhibitor for *L. monocytogenes* on RTE products; employ either a post-lethality treatment or a growth inhibitor for the pathogen on RTE products and/or; increase product and environmental testing including the employment of sanitation measures only.

The World Health Organization (WHO) working group (1988) on foodborne listeriosis proposed that food processing and retail food facilities as well as homes should separate non-contaminated foods from contaminated food; limit the potential for *L. monocytogenes* proliferation by avoiding unnecessary use of water and by employing adequate sanitation principles, including application of good agricultural and manufacturing practices as well as HACCP; and limiting vectors for *L. monocytogenes* transmission. Suitable designs of food processing and food handling equipment, suitable sanitation procedures and minimization of contamination from food contact surfaces could also help eliminate cross-contamination from the food processing environment. Simple practices like separating raw and cooked products, proper application of sanitizing and disinfection processes, such as pasteurization and cooking, as well as maintaining good hygiene practices will eliminate *L. monocytogenes* from food products (WHO 1988).

Several methods are used to control *Listeria* in food products. Doyle (1999) conducted a survey of a variety of techniques used to control *Listeria* in meat. These techniques include the use of organic acids and other preservatives, bacteriocins, thermal processes, irradiation, modified atmosphere packaging, high pressure, pulsed electric fields and electrolyzed oxidizing water, ultraviolet light, and ultrasound. Some of these techniques may not be economically feasible in a delicatessen environment. Moreover, bactericidal additives cannot be used in all food products, and little is known about the fate of *L. monocytogenes* on different types of deli meat contaminated by such processing equipment as meat slicers (Lin *et al.* 2006). Cost effective techniques aimed at minimizing *L. monocytogenes* cross-contamination of RTE meats by deli meat slicers are, therefore, necessary (Crandall *et al.* 2010).

Thermal inactivation

Thermal inactivation of bacteria can be achieved by the application of either dry or moist heat (Rahn 1945. The former refers to inactivation by oxidation, which involves denaturing of cell enzymes and proteins, whereas the latter refers to inactivation by coagulation of essential proteins in the cell (Russell 2003). Dry heat has been applied in the sterilization of glassware and certain instruments and materials. Examples of dry heating include direct flaming such as in sterilizing inoculating loops and needles by heating until metal has a red glow; incineration, which is an effective way to sterilize disposable items such as biological waste; and hot air sterilization, which involves placing objects in an oven and allow them to be sterilized, e.g. for 2 hours at 170^{o}C for sterilization. At higher temperatures, bacteria die more rapidly, and the cause of gradual increase in the death rate is believed to result from denaturing of essential enzymes and proteins in the cells due to either oxidation or coagulation (Rahn 1945, Russell 2003, Lado and Yousef 2007). The death rate is gradual and at no point does it increase abruptly. The temperature coefficients of microbial death by dry heat are very low, i.e. the relative change or reduction in the number of bacteria when the temperature is increased by one degree is lower for dry heat (Rahn 1945). In some cases moist heat is used to heat foods in the canning process, milk, beer and wine during pasteurization, culture media for bacteria, and surgical dressings (Rahn 1945). When *L. monocytogenes* is heated at temperatures above 56°C the result is ribosomal damage, protein unfolding and denaturation as well as enzyme inactivation. Ribosomal damage involves the loss of Mg^{+2} which leads to the separation of the more heat susceptible 30S and the lesser heat vulnerable 50S ribosomal subunits. Denaturation of the 30S ribosomal subunit is believed to be the main cause of bacterial death (Lado and Yousef 2007). Russell (2003) also reported that high temperatures are likely to affect, to some extent, every cellular component; including outer layers, membrane, enzymes, proteins, DNA and RNA and that a reduction in microbial population cannot be attributed to a single change within an organism. However, some of the damages may be more pronounced than others and they may be reparable depending on the degree of the temperature applied.

The heating power of steam (moist heat) is reported to come mainly from its latent heat of vaporization, which is the amount of heat energy required to convert boiling water to steam (Wilhelm *et al.* 2004). This amount of heat is large compared to that required to make water hot. In fact, the latent heat of vaporization is about seven times more than the latent heat of fusion (heat energy required to freeze water into ice or thaw water) (Wilhelm *et al.* 2004). For example, it takes 80 calories to make 1 liter of water boil, but 540 calories to convert that boiling water to steam.

Thermal inactivation kinetics

The time needed to destroy 90% of the micro-organisms or to reduce their numbers by a factor of 10 is referred to as the decimal reduction time or D-value. D-values differ for different microbial species and a higher D-value indicates greater heat resistance (Fellow 2009). Russell (2003) defined the D-value as the time, usually in minutes, necessary to produce a 1-$\log_{10}$ reduction in viability or to reduce the microbial population in colony-forming units (cfu) by 90% (to 10%) at a given temperature (Fig. 3). This, therefore, implies that the higher the number of micro-organisms present in a raw material, the longer it takes to reduce the numbers to a specified level (Fellows 2009). Moreover, microbial destruction occurs at a logarithmic rate, which means that theoretically a sterile product cannot be produced with certainty no matter how long the process time. It is for this reason that commercial processing methods target predetermined microbial reduction levels (Fellows 2009). The decimal reduction time is inversely related to temperature and as such cells die more rapidly at higher temperatures. The z-value (Thermal Death Time, TDT) is defined as the number of degrees (°C) to achieve a 10-fold reduction in the D-value (Fig. 4) or simply the temperature difference required for the thermal inactivation curve to cause one logarithmic reduction (Russell 2003, Fellows 2009). The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as thermal death time (TDT). Death rate is directly proportional to the concentration of microorganisms at any given time. Increasing the temperature decreases TDT, and lowering the temperature increases TDT. It can be obtained from either the slope of the semi-logarithmic thermal death time (TDT) curve (which is constructed by plotting D-values at different temperatures), or by using the temperature coefficient

formula $Q_{10} = 10^{10/z}$, where $z = 10/\log Q_{10}$, and Q_{10} is the temperature coefficient per 10°C rise in temperature (Russell 2003).

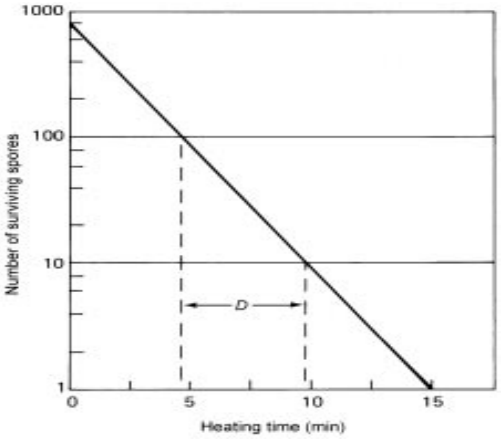

Fig. 3 Death rate curve (Fellows 2009).

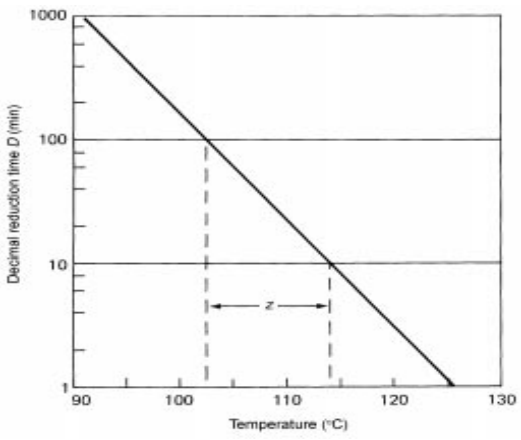

Fig. 4 TDT curve - Microbial destruction is faster at higher temperatures (e.g. 100 min at 102.5°C has the same lethal effect as 10 min at 113°C) (Fellows 2009).

The D-value and z-value are used to characterize the heat resistance of a micro-organism and its temperature dependence, respectively. Fellows (2009) compiled heat resistance values of selected pathogens. The D-values of *L. monocytogenes* in milk and meat products range between 0.22 and 0.58 min at 63.3°C and 1.6-16.7 min at 60°C, respectively.

Microbial inactivation has been traditionally assumed to follow the first order kinetics; i.e. the logarithmic reduction of bacteria is linear over time, at a given temperature. However, Stringer *et al.* (2000) argued that this is not justified, on a theoretical basis, because in most cases there are deviations which take the form of shoulders or tails. Different types of survival curves have been studied in terms of microbial death using mathematical models (Fig.5) (Peleg and Cole 1998, Peleg 2000, 2002, 2003. The failure to follow first-order kinetics may be attributed to limitations of the experimental procedure or the deviations may be an inherent feature bound to the mechanism of heat inactivation or resistance of the

microbial population. Russell (2003) stated that the deviations from log-linear kinetics in survival curves, which are an indication of different types of response to high temperatures, may be associated with heterogeneity of resistance within a microbial population or with changes in resistance during heating.

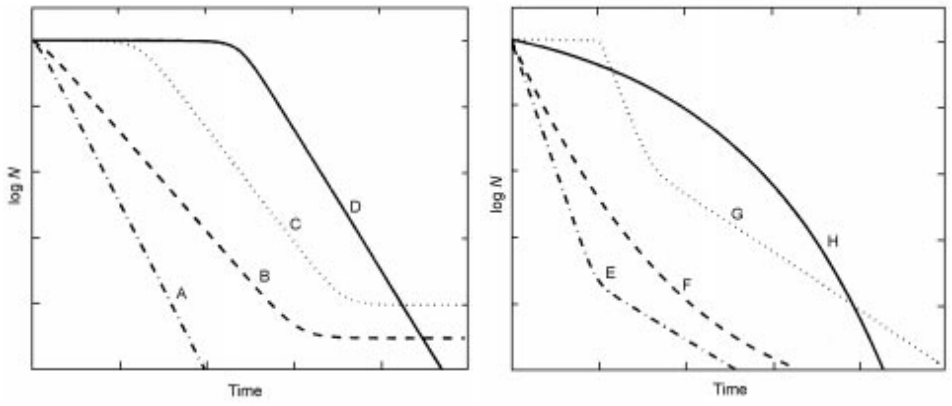

Fig. 5 Types of microbial inactivation curves: A, linear curve; B, linear curve with tailing; C, sigmoidal-like curve; D, curve with a shoulder; E, biphasic curve; F, concave curve; G biphasic curve with a shoulder; H, convex curve (Fellows 2009).

An initial shoulder on the curves may signify clumping of cells or demonstrate that inactivation occurs repeatedly on target sites. Tailing could indicate the presence of small numbers of large clumps or of heterogeneity of resistance in the population. A sigmoidal curve could result from a combination of factors, such as repeated inactivation on intracellular targets or it could be a result of heat adaptation of the pathogens during the heating process. Such deviations are known to occur more frequently with vegetative bacteria (Gould 1989, Fellows 2009). Crandall *et al.* (2010) reported that it was not possible to compute D- and z-values for *L. innocua* on the slicer materials because of the non-linear nature of the curves obtained.

Factors affecting thermal inactivation of bacteria

Most thermal inactivation studies have been conducted with food matrices (Bunning *et al.* 1998, Murphy *et al.* 1999, 2000, 2001a, 2001b, 2002, 2004). Thermal inactivation, such as pasteurization, is the most commonly used method of food preservation. *L. monocytogenes* is relatively resistant to most

inactivation methods compared with most non-spore forming organisms (Lado and Yousef 2007). Several factors influence bacterial inactivation (Russell 2003). Bacterial inactivation may be influenced by the composition and pH of the growth medium (Russell 2003). For example, Quintavalla *et al.* (1998) heated three strains of *Escherichia coli* in TSB and in pork slurry with curing agents at 60°C and the D-values ranged from 0.28 to 0.33 min and from 0.94 to 1.05 min, respectively, indicating that cells in pork slurry were more resistant than those in TSB. Inactivation may also be influenced by the type of organism, for example, there could be a strain-dependent response (Russell 2003). Growth conditions, such as the media, growth phase and temperature, among others, may also affect bacterial inactivation (Russell 2003). Heat resistance may be greater at the stationary than the logarithmic growth stage (Russell 2003). The heating method may also have an effect on Microbial inactivation (Russell 2003). Open heating systems are reported to be less accurate than closed ones (Russell 2003). A heating method that avoids local temperature variations is most preferred because a subpopulation of cells exposed to a lower temperature will appear to be more heat resistant and survival curves will tend to exhibit tailing (Russell 2003). Recovery conditions are also crucial in microbial inactivation (Russell 2003). For example, incubation conditions, after heat treatment, and the duration of the incubation period have been found to have an effect on the measured heat resistance of cells (Stringer *et al.* 2000). When Farber and Brown (1990) heat shocked *L. monocytogenes* in meat at 48^0C for 30 min before being heated at the final test temperatures, D-values at 62 and 64°C did not show any significant differences compared with control cells. However, upon increasing the heat shock time from 30 to 120 min, a significant increase in the $D_{64°C}$ value was observed. In addition, microbial enumeration on pure plates has been shown to increase measured heat inactivation compared with spread plates, suggesting that recovery of heat-stressed cells was greater with the spread plate method than with the pure plate method (Czechowicz *et al.* 1996).

Disinfection of food contact surfaces

The FDA Food Code (2009) requires that food-contact surfaces and equipment used for potentially hazardous foods be cleaned as needed throughout the day at least every 4 hours to prevent the growth of microorganisms on such surfaces. The Code also requires sufficient time for hot water or

chemicals to contact utensils or food-contact surfaces to destroy pathogens that may remain on surfaces after cleaning. It further requires surface temperatures of utensils being sanitized using hot water to reach 71°C (160°F). The actual temperatures should be consistent with the machine manufacturer's operating instructions and within limits specified in the Code. Sanitary food-contact surfaces can be attained by removing soil deposits and subsequently applying a sanitizer or disinfectant to reduce the number of residual microorganisms (Holah et al. 1998). Pre-cleaning is an important step in a sanitization program as some debris, such as organic matter, may inactivate or lead to decreased effectiveness of disinfectants (Holah et al. 1998). Mechanical cleaning prior to disinfection increases disinfectant efficacy, and in some cases, increases microbial log reductions (Holah *et al.* 1998). Effective regular cleaning can be more important than use of a disinfectant for reducing bacterial concentrations. Tebbutt (1984) reported that cleaning and rinsing alone may achieve 2 to 3 log reduction. Crandall *et al.* (2012) demonstrated that sanitizers were able to remove 2 to 3 log CFU/cm^2 of inoculated *Listeria* cocktail attached on stainless steel. There is a wide range of disinfectants for use on food-contact surfaces in the food industry including those which contain chlorine compounds (e.g., bleach), peroxide and peroxyacid mixtures, carboxylic acids, quaternary ammonium compounds, acid anionic, and iodine compounds (Gaulin *et al.* 2011).

Thermal treatment of deli meat slicers

A deli slicer is constructed of, among others parts, numerous stainless steel and aluminum components, which may or may not be easily disassembled. These components may become eroded with continued washing and through the use of strong chemical sanitizers (CCDR, 1998). The slicer seams, seals, gaskets, eroded metal surfaces and other hard-to-reach areas may accumulate liquids and food debris as well as harbor *L. monocytogenes* and other pathogens if they are not properly maintained. Cleaning and sanitizing methods, over and above the conventional methods, are necessary to minimize *L. monocytogenes* cross-contamination of RTE products by the deli meat slicer in the deli environment (Crandall *et al.* 2011).

An attempt was made by Crandall *et al.* (2010) to inactivate *L. innocua* (a surrogate of *L. monocytogenes*) on deli slicers using existing delicatessen ovens to provide lethal dry heat treatment.

They inoculated stainless steel and cast aluminum components of a deli slicer and coupons, cut from a deli slicer, with *L. innocua* and then subjected these materials to different time-temperature treatments in an oven. The idea was to emulate the use of bread proofer ovens, where by the heat does not reach the typical dry heat sterilization temperatures of 160 to 180^{o}C but still yield a significant reduction in microbial population. This study also assessed the difference in survival of *L. innocua* on the slicer's stainless steel blade versus the cast aluminum guard coupons. The results indicated that dry thermal treatment at 80^{o}C for up to 15 h was not sufficient to achieve a 5-log reduction of residual *L. innocua* that may have survived improper cleaning and sanitizing of the deli slicer. There was also no significant difference in *L. innocua* reduction between the two types of metal coupons. In another experiment Crandall *et al.*. (2009) established that subjecting deli slicer components to 82°C for 3 hours under saturated moisture conditions (100% relative humidity) could produce a 5 log reduction of the more heat resistant *L. innocua*. Murphy *et al.* (2001) detected five to three $\log_{10}$ (cfu/g) of *Listeria* in patties after the chicken patties reached a final center temperature of 70 to 80°C; however, in a different study Murphy *et al.* (1999) observed a 7 $\log_{10}$ (cfu/g) reduction for *Listeria* when chicken breast meat was cooked to 70°C in a water bath. They concluded that because, in the former study, steam was not introduced, the relative humidity in the air convection oven was low (6 to 10%). Harrison and Carpenter (1989) were able to achieve a 1.8 to 2.3 log reduction and 4 to 5.6 log reduction after cooking chicken samples up to 65.6-71.1°C and 73.9, 76.7 and 82.2°C, respectively, even though the heating time(s) are not clearly stated in this study. Crandall *et al.* (2010), therefore, proposed a study that would exploit the use of moist heat at 66 and 80°C on a contaminated slicer. It has been reported in other studies that dry heat is a far less effective heat inactivation process than moist heat (Rahn 1945, Russell 2003). Concerns exist, however, with regards to the effects on the durability and safety of the slicer and its electrical components following repeated application of a moist heat inactivation process and an extensive hot drying cycle, aimed at eliminating *L. monocytogenes* from the deli slicers in a retail deli setting. Moreover, there is need to determine the heat transfer rates from a commercial convection oven or moist-heating bread

proofer cabinet into the potential *L. monocytogenes* harborages on a commercial deli slicer (Crandall *et al.* 2011).

Viable versus viable-but-non-culturable (VBNC) *L. monocytogenes* cells

The VBNC state of bacteria has been defined as the inability of bacteria to be cultured on media which normally supports its growth, and yet they still exhibit indicators of metabolic activity. Bacteria in this state have been reported to be capable of being revived to a metabolically active state when the conditions permit (Oliver 2000). This "non-recoverable" stage of existence adopted by bacteria in their normal culture media was first described by Xu *et al.* (1982) in their study involving *Escherichia coli* and *Vibrio cholerae* in the aquatic environment. The VBNC state is believed to be a survival strategy adopted by bacteria when they are exposed to stressful environmental conditions such as nutrient depletion, temperatures outside the optimal range of growth, elevated osmotic concentrations such as seawater, oxygen concentration, or exposure to white light (Oliver 2005, Rice *et al.*, 2000). These conditions are considered to be otherwise lethal if the organism did not enter into this dormant state (Oliver 2005). Characteristics of VBNC cells include apparent cell integrity, exhibition of some form of measurable metabolic activity and the apparent ability to be resuscitated when conditions permit (Kell *et al.* 1998). Several studies have been undertaken to investigate this condition in various pathogens, including *L. monocytogenes*, *Escherichia coli*, *Citrobacter freundii*, and *Vibrio vulnificus*, among others (Boulos *et al.* 1999, Li *et al.* 2003, Lindback *et al.* 2010). A long list of bacteria and pathogens that enter into the VBNC state has been compiled by Oliver (2005) and Oliver (2010), respectively. However, only a few gram-positive bacteria including *L. monocytogenes* have been reported as having the potential to enter the VBNC state (Byrd *et al.*. 1991, Besnard *et al.*2000, Li *et al.* 2003). Li *et al.* (2003) found that refrigeration temperature (4°C) and carbon dioxide, normally used in preservation and modified atmosphere processes, respectively, do not induce the VBNC state in *L. monocytogenes*. This could be explained by the characteristic psychrotrophic and facultative nature of *L. monocytogenes*, respectively. However, Lindback *et al.* (2010) induced the VBNC state in *L. monocytogenes* Scott A by starving the pathogen in microcosm water at 4°C for 12 weeks. Cappelier *et al.* (2005) discovered that *L.*

monocytogenes strains, including the Scott A strain, when incubated in 20°C and 4°C microcosm water, respectively, became VBNC, but not infectious when analyzed using the human adenocarcinoma cell line (HT-29) and a mouse model. This suggests that *L. monocytogenes* strains have the potential to remain in the aquatic environment for a long time in the presumably non-pathogenic VBNC state. As already discussed, cells which enter the VBNC state tend to protect themselves from environmental stresses which may potentially result to an increased resistance even to sanitation methods particularly in the food processing industry. Gunasekera *et al.* (2002) demonstrated that a large number of VBNC *Escherichia coli* and *Pseudomonas putida* cells in pasteurized milk were metabolically active after pasteurization for 30 min at 63.5°C even though they were not able to form colonies. The fact that VBNC cells cannot be detected by traditional culturing methods yet they are capable of resuscitation and growth into the culturable state under favorable conditions makes them potential threats to public health (Byrd *et al.*. 1991, Besnard *et al.* 2000, Oliver 2010). It has also been reported by Besnard *et al.* (2000) and Li *et al.* (2003) that *L. monocytogenes* cells tend to be elongated when they enter the VBNC stage, due to continued absorption of nutrients. This characteristic has been used to differentiate between viable/dead and VBNC *L. monocytogenes* cells (Besnard *et al.* 2000).

Several methods have been used to determine the presence of viable/dead and VBNC cells in environmental and food samples (Keer and Birch 2003, Rudi *et al.* 2005, Dreux *et al.* 2007). These methods can be grouped into three categories based on the approach used in the assessment of bacterial viability. For example, metabolic activity responsiveness including direct viable count test, detection of respiration and detection of mRNA synthesis; presence of nucleic acid (DNA, mRNA, and rRNA) including PCR (Nogva *et al.* 2000), reverse transcriptase (RT)-PCR (Ye *et al.* 2011), Cytochemical staining, nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), reverse transcriptase-strand displacement amplification (RT-SDA), and hybridization; as well as cellular integrity including, membrane potential, membrane integrity (e.g., BacLight Live/Dead assay), and flow cytometry (Keer and Birch 2003, Oliver 2010). Other methods include inhibition by nalidixic acid (Kogure procedure based on cellular elongation in the presence of DNA gyrase inhibitors) and

radiolabelling (Besnard *et al.* 2000); Direct Viable Count (DVC) and 5-cyano-2,3-ditolyl tetrazolium-4,6-di-amidino-2- phenylindole (CTC-DAPI) double staining methods (Cappelier *et al.* 2005); ethidium monoazide bromide (EMA)-PCR (Rudi *et al.* 2005); quantitative PCR (qPCR) (Nogva *et al.* 2003, Rantsiou *et al.* 2008); for heterotrophic cells (HPC) 2-(p-iodophenyl)-3-(p-nitrophenyl)-5- phenyl tetrazolium chloride (INT) is converted to INT-formazan resulting in red spots (Besnard *et al.* 2000); acridine orange direct count (AODC); 4,6-di-amidino-2- phenylindole (DAPI) direct count; and rhodamine 123 (Rh123) (Boulos *et al.* 1999). Some of these methods lack specificity (Boulos *et al.* 1999).

The purpose of this study was to: assess the effectiveness of selected cleaning cloths in cleaning food contact surfaces; determine the effectiveness of cloth-sanitizer combinations in reducing food contact surface contamination to acceptable levels; and determine if the detection capability of the two testing methods (ATP-B, and TPC) was the same. It also aimed at determining the optimum moist heat treatment and moist heat and sanitizer combination necessary to significantly reduce *Listeria* strains on deli slicer components and to rule out the presence of VBNC cells after the moist heat treatment.

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CHAPTER III

CLEANING AND DECONTAMINATION EFFICACY OF WIPING CLOTHS AND SILVER DIHYDROGEN CITRATE ON FOOD CONTACT SURFACES

ABSTRACT

Food contact surfaces present high risks for cross-contamination and are implicated in the transmission of foodborne pathogens. The study aimed at testing the efficacy of four wipe cloths types (cotton bar towel, non-woven, microfiber, and blended cellulose/cotton) with quaternary ammonia or silver dihydrogen citrate (SDC) in cleaning food contact surfaces. Swab samples collected from untreated, cloth and cloth-sanitizer treated surfaces were subjected to hygiene monitoring using adenosine triphosphate (ATP) bioluminescence and aerobic total plate counting (TPC) assays. ATP measurements taken after wiping the surfaces showed poor cleaning by non-woven cloths (2.89 RLU/100 cm^2) than the microfiber (2.30 RLU/100 cm^2), cotton terry bar (2.26 RLU/100 cm^2) and blended cellulose/cotton cloth types (2.20 RLU/100 cm^2). The cellulose/cotton cloth showed highest log reduction of ATP-B RLU values (95%) and CFU values (98.03%) when used in combination with the SDC sanitizer. Cleaning effect of wiping cloths on food contact surfaces can be enhanced by dipping them in SDC sanitizer. ATP-B measurements can be used for real-time hygiene monitoring in public sector and testing microbial contamination provides more reliable measure of cleanliness. Contamination of food contact surfaces need regular hygiene monitoring and this study could help to estimate and establish contamination thresholds for surfaces at public sector facilities and to test the effectiveness of cleaning methods.

Keywords: Disinfection, Environmental health, Food Safety, Microbial contamination

INTRODUCTION

Cross-contamination of food contact surfaces is a major safety concern for food-service facilities where any inefficient cleaning with reusable wiping cloths could spread harmful bacteria and viruses posing potential serious health hazards to consumers. Nearly 80% of the reported foodborne outbreaks can be traced back to food-service facilities with major contributing factors being time/temperature abuse of prepared foods, poor personal hygiene of the food preparer, and cross contamination in the food-service establishments (Collins 1997). Thus, microbiological quality evaluation of commercial food preparation and serving surfaces in food-service establishments is critical to public health. A significant reduction in bacterial load and improved hygiene conditions of such areas can be achieved by use of efficient cleaning cloths and disinfectants. Regular monitoring for the effectiveness of cleaning practices could serve as a crucial preventative measure for potential foodborne outbreaks. From the point of food production and preparation to consumption, there could be several opportunities for food to become contaminated. Food contact surfaces have been predominantly incriminated in the contamination of food products in the food industry (Tebbutt 1984, Evans *et al.* 1998). Although cleaning is regularly practiced in food-service establishments, there is great reliance upon visual assessment for hygiene monitoring, which is not reliable in determining the potential risk constituted by the contaminated food contact surfaces (Tebbutt *et al.* 2007). Evidently, the data pertaining to the presence of foodborne pathogens on surfaces of food-service establishments and their potential to spread from surfaces to food is justification enough to ensure effective cleaning regimes. The microbiological assessment of food contact surfaces is therefore necessary to determine the effectiveness of cleaning methods on surfaces (Verran *et al.* 2002).

The presence of food residues on a food contact surface indicates that the surface has not been adequately cleaned and the constituent food particles may provide nutrients for the consequent growth of microorganisms (Leon and Albrecht 2007) that may also be potentially pathogenic. Some studies have recommended periodic microbiological assessment of high-risk food establishments to reduce the risk of foodborne disease outbreaks (Moore and Griffith 2002). However, microbiological methods such as the

aerobic plate count take up to 48 hours to give feedback on findings (Leon and Albrecht 2007). The efficacy of sampling methods and rapid assay methods for evaluating the cleanliness of food contact surfaces has been examined in several studies (Corbitt *et al.* 2000, Griffith *et al.* 2000, Larson *et al.* 2003, Matticka *et al.*2003, Chen and Godwin 2006, Hong and Brown 2009). Among several test methods, ATP bioluminescence (ATP-B) assay is one of the widely used methods for the detection of microbial contamination and food residues in the food industry (Corbitt *et al.* 2000, Lappalainen *et al.*2000, Larson *et al.* 2003, Chen and Godwin 2006, Powitz 2007). This method provides a real-time estimation of total surface cleanliness including the presence of organic debris and microbial contamination (Leon and Albrecht 2007). However, conflicting findings have been reported with regard to correlation between ATP bioluminescence and the number of bacteria in a sample tested using the TPC method (Tebbutt *et al.* 2007, Whitehead *et al*. 2008). Hence, the ATP-B assay is used as an indicator of total cleanliness and unlike the TPC method; it does not quantify the number of microorganisms in a sample. Moreover, any trace of food residues may carry ATPs adding to the amount of microbial ATP. This should explain the reason why some studies have found poor correlation between ATP results and the number of bacteria in samples.

Selecting an effective combination of both cleaning materials and disinfectants is essential in ensuring the clean or hygienic food contact surfaces (Diab-Elschahawi *et al*. 2010). Even though the use of cleaning cloths and disinfectants is an important part of the cleaning process, reusable cleaning cloths usage is discouraged because of their ability to re-contaminate surfaces; however, food premises have continued to use them (Tebbutt 1991). Nonetheless, there is very little information on the effectiveness of commonly used cleaning materials in the deli environment. Studies have been conducted to determine the effectiveness of cleaning regimes (Tebbutt 1984, 1991, Griffith *et al*. 2000, Worsfold 2001) but, with little focus on the efficacy of individual cleaning cloths. The use of cleaning cloths in combination with disinfectants has also been investigated (Tebbutt 1991, 2007). Disinfectants are used to reduce the number of potential pathogens on food contact surfaces and are widely accepted to ensure food safety if properly used. Surfaces cleaned with a cloth soaked in a disinfectant are more likely to be successfully cleaned

than those wiped with cloths which are not disinfected after use (Tebbutt 1991). To achieve maximum cleaning effect, cleaning cloths in most food-service establishments are usually soaked in dilute disinfectants whenever they are not in use. However, a majority of retail food service establishments fail to prepare correct dilutions of disinfectants and do not change them until they are deactivated resulting to bacterial contamination of the cloths (Tebbutt 1988). Such contaminated cloths are difficult to adequately disinfect after each use (Tebbutt 1991). Silver Dihydrogen Citrate (SDC) spray is a ready to use disinfectant which has broad spectrum and residual disinfectant characteristics against bacteria, fungi, and viruses. It contains silver ions stabilized in citric acid and known to damage an organism's DNA and protein structure by lysing cell membrane, causing the cell death (Purebio 2012). We tested for the efficacy of four different wiping cloth types along with SDC or quaternary ammonia in cleaning food contact surfaces. The efficacy testing of SDC spray to reduce microbial counts on test surfaces was included in this study as the post wiping use of disinfectant sprays may contribute in the reduction of cross-contamination caused by inefficiently sanitized cleaning cloths. Thus, the study intended to assess the effectiveness of commonly used cleaning cloths in cleaning food contact surfaces; determine the effectiveness of cloth and disinfectant combinations in reducing food contact surface contamination to acceptable levels; and correlate the detection capability of ATP-B and TPC testing methods.

MATERIALS AND METHODS

Wiping cloths and treatments

Four different wiping cloths selected in this study were; blended cellulose/cotton cloth (70% cellulose and 30% cotton, supplied by Kalle USA, Gurnee, IL), non-woven wipes (50% viscose and 50% polyester, supplied by Ecolab, St. Paul, MN), microfiber cloth (supplied by Super Detail, Inc. San Diego, CA.), and cotton terry bar towels (100% cotton utility dishcloth supplied by Mainstays, Pakistan). Quaternary ammonia cleaning (QAC) solution (Oasis 146 Multi-Quat sanitizer, Ecolab Inc., St Paul, MN) and Silver Dihydrogen Citrate (SDC) disinfectant (Pure Bioscience, El Cajon, CA) were also used. Formica dining tables under daily use at a local operating food service facility were selected as the representative food contact surfaces of public sector. A no cloth positive control was included along with the cloth and cloth-disinfectant combination treatments.

Quaternary ammonia treatment of food contact surfaces and sampling procedure

In this part of our study, the cleaning crew members of the dining facility were given with the above four cloth types for cleaning the Formica dining tables. The cloth treatment involved use of cloths dipped in quaternary ammonia and squeezed thoroughly before wiping. The tables in the premises were cleaned by following the set procedure of the establishment which included placing the cloths into a quaternary ammonia solution while the cloths were not being used. We asked the cleaning personnel to use each type of cloth over a period of one week for wiping the table surfaces. A different test cloth was used for each of the following weeks, until all of the four cloth types were tested. For each cloth type, 6 tables were randomly selected and 5 replicate samples from each table were collected after wiping. This was repeated for three days to collect a total of 360 samples for all 4 cloths. Sampling was done on 4^{th}, 5^{th}, and 6^{th} days after the introduction of a cleaning cloth to provide sufficient time to avoid prior cloth's cleaning effect. Sample collection was done by swabbing a 100 cm^2 area of table surfaces within an aluminum square template. The swabbing was done using ATP swabs that come with the ATP-

bioluminescence assay kit and were subjected to hygiene monitoring as described later. Relative light units (RLU) were recorded for each test area.

Silver Dihydrogen Citrate (SDC) disinfectant treatment of food contact surfaces and sampling procedure

This second part of the study was done by using three types of cleaning cloths consisting of two cloth types (Blended cellulose/cotton cloth and Non-woven wipe) that performed best and worst, respectively in the first study, and a third cloth type (cotton terry bar towel) which is commonly used in the commercial food-service environment. No wipe positive control samples were collected before cloth treatments. The treatments were done by dipping a cloth in sterile water (Cloth type) or SDC disinfectant (Cloth-disinfectant), and squeezed thoroughly before wiping. The wiping treatments were carried out by a trained research staff that was designated to wipe the table surfaces throughout the study to ensure consistency and uniformity in the wiping method. Six randomly selected tables were assigned for each cloth type and a before wipe positive control, post wiping cloth and cloth-disinfectant samples were taken per table. This was repeated for three days in a week per cloth using different tables each day. Manufacturer recommended instructions were followed in using the SDC disinfectant wherein, it was sprayed on the table surfaces and gently wiped with clean terry cloth after 5 minutes to minimize dampness caused by the disinfectant and the sampling was done using sterile cotton swabs. This treatment was considered as a cloth-disinfectant treatment because the final microbial counts reflected the combined effect of both the terry cloth wiping and the disinfectant effect.

ATP bioluminescence (ATP-B) Assay

ATP samples were collected and measured using a SystemSURE Plus™ ATP Hygiene Monitoring System (Hygiena USA, Camarillo CA) as per the manufacturer's instructions (Hygiena, 2012). The ATP-B samples were collected with the provided Ultrasnap ATP test swab within a 100 cm^2 sampling area by swabbing in a zigzag horizontal and vertical pattern for 10 times each way, while rotating the swab and applying slight pressure on the surface being sampled. The tip of the swab handle was broken to release the luciferase/luciferin reagent, returned to its container in the device that was

shaken from side to side for about 5 seconds and allowed to stand upright for 15 seconds. Readings were taken and recorded in relative light units (RLU). An ATP-B test scale guidelines provided by the manufacturer were used as RLU >30 = dirty, RLU between 11 and 29 = caution and RLU<10 = clean, and this scale was used to measure the effectiveness of the cleaning cloth types in removing total surface contamination from study surfaces.

Aerobic Total Plate Count (TPC) Assay

Sterile calcium alginate swabs (Puritan Medical Products Company LLC, Guilford, Maine USA) were used to collect aerobic plate count samples from 100 cm^2 sampling areas adjacent to the area that was swabbed for ATP-B. The sampling procedure was similar to that for ATP-B but after sampling, the swabs were placed in sterile tubes containing 2 ml of 0.1% buffered peptone water (BPW) that were held cold on ice. The BPW tubes were taken to the lab within 2 h of the sample collection exercise, and thoroughly mixed to prepare serial dilutions of the samples. One ml of the diluted samples was plated onto 3M aerobic count petrifilms (3M Co., St. Paul, MN), and incubated for 24 h at 37°C . Recovered colonies were then visually enumerated and expressed as CFU/cm^2 as indicated below.

Data analysis

Both TPC and the ATP-B data were manually recorded in an Excel spreadsheet (Microsoft Corp., Redmond, WA) and were converted CFU/cm^2 and RLU/cm^2 respectively, using the following formulae;

$$\text{CFU/cm}^2 = \frac{\text{(Average CFU per table) x (Volume of original suspension)}}{\text{(Total surface area x number of swabs) x (Dilution factor)}} \quad (1)$$

$$\text{RLU/cm}^2 = \frac{\text{Average RLU per table}}{\text{Total surface area}} \quad (2)$$

The mean log CFU/100 cm^2 and mean log RLU/100 cm^2 recovered after cleaning and disinfecting surfaces with each cloth and cloth-disinfectant combinations were analyzed by analysis of variance using PROC ANOVA analysis in SAS 9.2 (SAS Institutes, Cary, NC). Means separations were compared by

least significant differences (LSD) where means with the same letter were not considered significantly different ($p<0.05$).

RESULTS

Cloth + Quaternary ammonia treatments

Results of using quaternary ammonia dipped cloth types for wiping the table surfaces are shown in table 1. The RLU values depict the amount of light produced depending on the level of contamination from the sample (higher the ATP numbers or higher luminescence means high level of contamination). We noticed some negative RLU values due to variability in contamination of certain sampling areas and those data points were not taken into consideration for calculations. The observed RLU values were log transformed for comparison and as shown in table 1, the non-woven wipe was the most poorly performing cleaning cloth type with the highest average mean log RLU (2.89 RLU/100 cm^2). Significant mean differences ($p<0.05$) were noted between the mean RLU values of non-woven cloth and all the other three cloth types. The RLU values of blended cellulose/cotton cloth and microfiber cloths also differed significantly. There were no significant mean differences between the cleaning effect of cotton terry bar and microfiber cloth types, and the cotton terry bar RLU values were on par with the blended cellulose/cotton cloth. However, the lowest RLU values of both the cotton terry bar and the cellulose/cotton cloth types suggested that their cleaning effect was superior in performance with other two cloth types. No plate counts were measured from this part of the study.

Cloth treatments

The ATP-B levels measured as RLU values/100 cm^2 after wiping the table surfaces with each of the blended cellulose/cotton, cotton terry bar, non-woven viscose/polyester cloth types (dipped in water and squeezed) were 169, 226 and 227 RLU/100 cm^2 respectively. When compared with the control (dirty) surfaces, the blended cellulose/cotton, cotton terry bar, and non-woven viscose/polyester cloth types could bring down the mean log reductions in RLU values by 28.05%, 25.10% and 18.09%, respectively. Further, there was no significant difference ($p>0.05$) in cleaning effect among the three cloth types (Fig.

1). Although the RLU log reductions after wiping with each cloth differed significantly ($p<0.05$) from the respective controls, the surfaces remained dirty with respective to the Hygiena ATP-B scale as these levels exceeded the acceptable RLU levels (Hygiena 2012).

The results of total plate counts (TPC) obtained from the samples collected after wiping the table surfaces with each of the three cloth types are presented in Figure 2. For the positive controls, the TPC values measured before wiping the table surfaces with cotton terry, non-woven viscose/polyester, and blended cellulose/cotton cloths were 104.9, 125.4, and 50.1 CFU/100cm^2 and were reduced by 67.08%, 68.20% and 76.74% in the mean log CFU/100 cm^2 respectively after wiping. However, there was no significant difference in the log CFU/100 cm^2 values or the cleaning effect on table surfaces among the three cloth types (Fig. 2).

Cloth + silver dihydrogen citrate (SDC) disinfectant treatments

Wiping the table surfaces with the cloth types dipped and squeezed in SDC disinfectant showed significant reductions in the ATP-B values and the total plate counts compared with the above treatments. The ATP-B levels measured after the cloth-SDC treatments were near or within the acceptable levels of <10 RLU based on the ATP-B scale at 12, 5 and 6 RLU for cotton terry bar, blended cellulose/cotton and non-woven viscose/polyester cloth-disinfectant combinations respectively and the corresponding mean log reductions were above 95% for all cloth types with no significant differences. However, the cleaning effect was significantly ($p<0.05$) improved with the use of disinfectant SDC with any cloth type when compared with the cloth alone treatments. This further suggested the cleaning efficiency of the tested cloth types can be enhanced when combined with the disinfectant spray as shown by the significant reductions in log RLU/100 cm^2 values (Fig. 1). Similar observations were noted with regard to the total plate counts wherein bacterial contamination was reduced to 0.7, 0.6, and 0.3 CFU/100 cm^2 for cotton terry, non-woven viscose/polyester and blended cellulose/cotton cloth-disinfectant combinations respectively, representing a corresponding reduction of 1.77 (95.70%), 1.66 (94.51%) and 1.54 (98.03%) log CFU/100 cm^2 (Fig. 2). Although the percent log reduction for blended cellulose/cotton cloth-

disinfectant combination was superior among all the cloth-disinfectant combinations, the mean differences of CFU/100 cm^2 were not significantly different ($p<0.05$).

DISCUSSION

As several types of wiping cloths and disinfectants exist in the market for cleaning food contact surfaces, it is essential for food service establishments to choose the best cleaning supplies that will not only clean the food contact surfaces but also reduce surface microbial loads to acceptable limits. This study assessed the effectiveness of selected cleaning cloth types and cloth-disinfectant combinations used in food service establishments to clean the food contact surfaces. The ATP-B measurements indicated that none of the tested cloth types when used alone, could bring the cleanliness to acceptable levels (RLU <10). Furthermore, the commonly followed cleaning practice in restaurants involving soaking of wiping cloths in quaternary ammonia for cleaning table surfaces also proved to be less effective in reducing contamination levels as seen by the RLU values. However, when the SDC disinfectant was used in the second study to sanitize the Formica surfaces, the contamination was reduced to acceptable levels for non-woven and cellulose/cotton cloth types as per the ATP-B scale. Although the total plate counts (TPC) for the dirty surfaces (positive controls) were less than 2.5 log CFU/cm^2, it is worth noting that low microbial levels on surfaces reduce the accuracy and reproducibility of all sampling methods (Tebbutt 1991). Moreover, the low bacterial counts can also be attributed to the fact that the tested table surfaces were subjected to daily cleaning with quaternary ammonia prior to the initiation of the study. The same cannot be said for the higher ATP-B measurements because the test detects adenosine triphosphates from both eukaryotic and prokaryotic cells. It should also be noted that the chemical components of a disinfectant may also interfere with the ATP-B fluorescence as revealed by some studies on the possibility of disinfectants reducing the activity of the luciferase enzyme and also acting as ATP-releasing agents, thereby contributing to potential false positive results (Lappalainen et al 2000, Powitz 2007). Hence the ATP-B measurements should not be considered as an alternative for the total plate count results, but should be confirmed by estimating the TPC counts as demonstrated in this study. For these

reasons, the table surfaces in this study would be considered “clean” with regards to the presence of microbial cells based on the TPC assay, but “dirty” based on the ATP-B test. In the case of cloth treatments, although the tested cloth types in this study did not differ significantly in their cleaning effects, the blended cellulose/cloth type yielded higher percent log reduction than others when used alone or in combination with the disinfectant or disinfectant. The log reduction levels in both RLU and CFU values were highest when the cloths were used with SDC disinfectant. In a different study where we compared cleaning fabrics for bacterial removal from food contact surfaces, we revealed that the blended cellulose/cotton cloth had the highest cleaning ability because its pores and thickness made it possible to trap bacteria (Koo *et al.* 2011). The cellulose/cotton cloth is a sponge-like cloth made of a blend of cellulose (70%) and cotton (30%) fibers. Glauber's salt (sodium sulfate) crystals are added in the fibers to enhance their absorption properties (Evans *et al*. 1998). These properties may have contributed towards the effectiveness of the cloth in this study.

During the study period, we noticed wide variation in the results for positive control from some of the table surfaces indicating the uneven contamination levels of the surfaces and hence, certain steps in sample collection protocol such as the pressure applied when cleaning food contact surfaces, swabbing method, the effectiveness of the swab in picking up microbial contamination and consequently dislodging into the buffered peptone water could limit the sensitivity of the swabbing method (Tebbutt 1991). Finally, the efficacy of the testing methods could also be another confounding factor. Thus, there is a need to establish contamination thresholds for food contact surfaces on which to base the effectiveness of cleaning methods. It would be expedient to have microbial criteria for testing cleanliness of food contact surfaces. The European Commission (2001) has recommended a guideline of less than 10 CFU/cm^2 for cleaned and disinfected surfaces in meat establishments. Other studies are recommending microbial levels less that 2.5 CFU/cm^2 for clean and sanitized surfaces (Worsfold and Griffith, 2001, Tebbutt *et al*. 2007). Since the criteria used to determine the scales that come with the ATP-B test kits is not verified in this study, we followed the suggestion of Worsfold and Griffith (2001), who have stated that an ATP value of 500 RLU being would be equivalent to less than 2.5 CFU/cm^2 for a surface to be considered clean. Thus,

based on the results of this work and the findings of other studies as discussed earlier, the standard criteria for acceptable microbial levels on food contact surfaces may range between <2.5 CFU/cm^2 and 10 CFU/cm^2 depending on the usage of the surfaces being evaluated. Although it may not be easy to tell the level of contamination that may constitute a health risk (Tebbutt *et al*. 2007) unless specific pathogen tests are done, the microbial thresholds for clean surfaces would help food service establishments to determine the efficacy of their cleaning regimes as well as to reduce the potential risk of spreading foodborne diseases.

In conclusion, we demonstrated that it is crucial for food service establishments to make appropriate choices of cleaning and hygiene monitoring. The use of cleaning cloths should be done in combination with an effective disinfectant when cleaning food contact surfaces. Our results are in agreement with the findings made by Tebbutt (1998) who revealed that the combination of a disinfectant and physical removal of microbes with cleaning cloths is essential. TPC and ATP-B data displayed comparable trends of contamination levels suggesting that either method could be used to determine the effectiveness of cleaning methods on food contact surfaces. The ability of the ATP-B test producing real-time results could play a significant role in providing quick verifications for total surface cleanliness estimates including the presence of organic fragments and microbial contamination. After potential contamination has been detected, corrective measures could then be implemented in time before food is served or distributed. The use of ATP-B should not, however, replace the TPC method but should be used as a complementary method to provide quick confirmation of bacterial contamination on food contact surfaces.

ACKNOWLEDGMENTS

We are thankful to KALLE USA, the United States Department of Agriculture (USDA), American Meat Institute (AMI), and the National Integrated Food Safety Initiative (NIFSI, grant number 2010-51110-21004) for funding the study.

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TABLES

Table 1 LSD values showing differences in mean log RLU/100 cm^2 for each cloth type in the first study[a].

Cloth types	N[b]	ATP-B Test Mean Log RLU/100cm^2
Non-woven	59[†]	2.89 ± 0.30^{A}
Microfiber	90	2.30 ± 0.30^{B}
Cotton Terry	88[†]	2.26 ± 0.25^{CB}
Cellulose/cotton	90	2.20 ± 0.28^{C}

[a]Means with the same letter notation are not significantly different

N[b] is the number of samples collected per treatment

† N differs for some cloth types because negative values were removed

(Negative values due to variability in contamination of sampling area were not included)

FIGURE LEGENDS

Figure 1 ATP-B results in Relative Log Units (RLU)/100 cm^2, indicating the efficacy of cleaning cloth types and cloth-disinfectant combinations in reducing total surface contamination. The RLU values for no wipe control, cloth alone and the cloth-SDC disinfectant combinations are shown. The ATP measurements are expressed in mean log RLU and the same letter on the bar graphs indicates no significant statistical difference in the RLU values. Error bars represent the standard deviation of ATP-B measurement conducted in triplicate for each table surface sampled.

Figure 2 TPC results in CFU/100 cm^2, indicating the efficacy of cleaning cloth types and cloth-disinfectant combinations in reducing microbial counts from food contact surfaces. The results for no wipe control, cloth alone and the cloth-SDC disinfectant combinations are shown. The CFU measurements are expressed as mean log CFU/100 cm^2 where same letter on the bar graphs indicates no significant statistical difference in the CFU values. Error bars represent the standard deviation of ATP-B measurement conducted in triplicate for each table surface sampled.

Figure 1

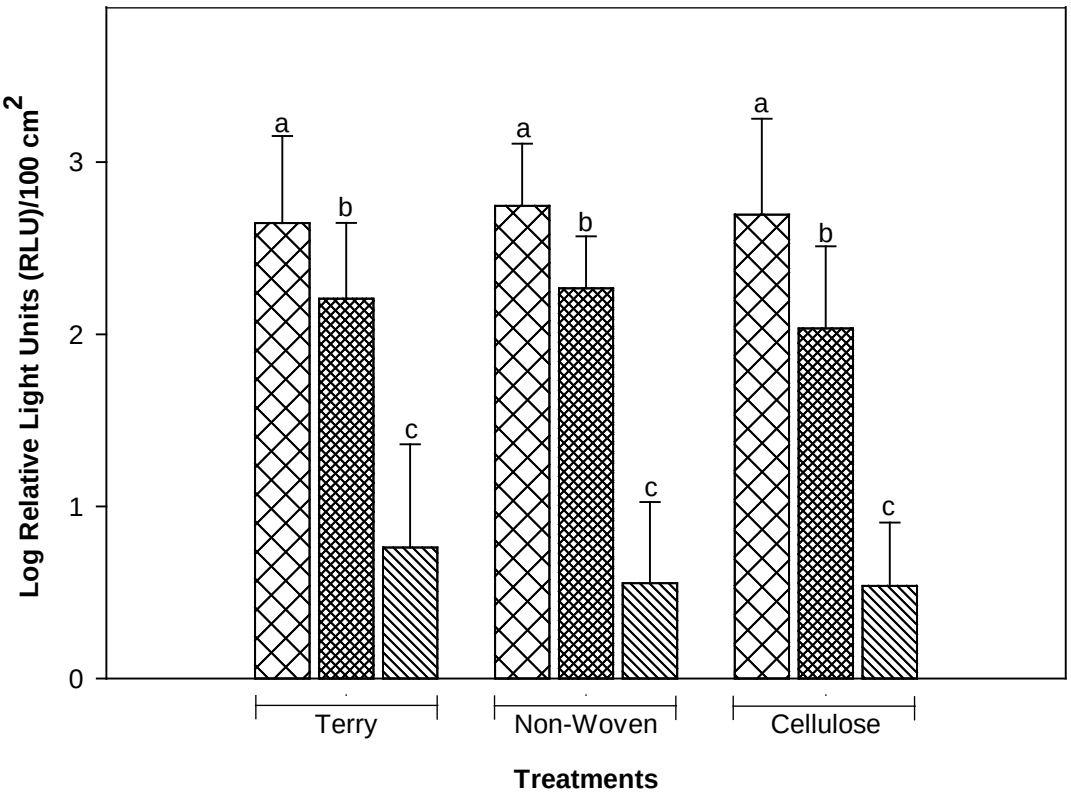
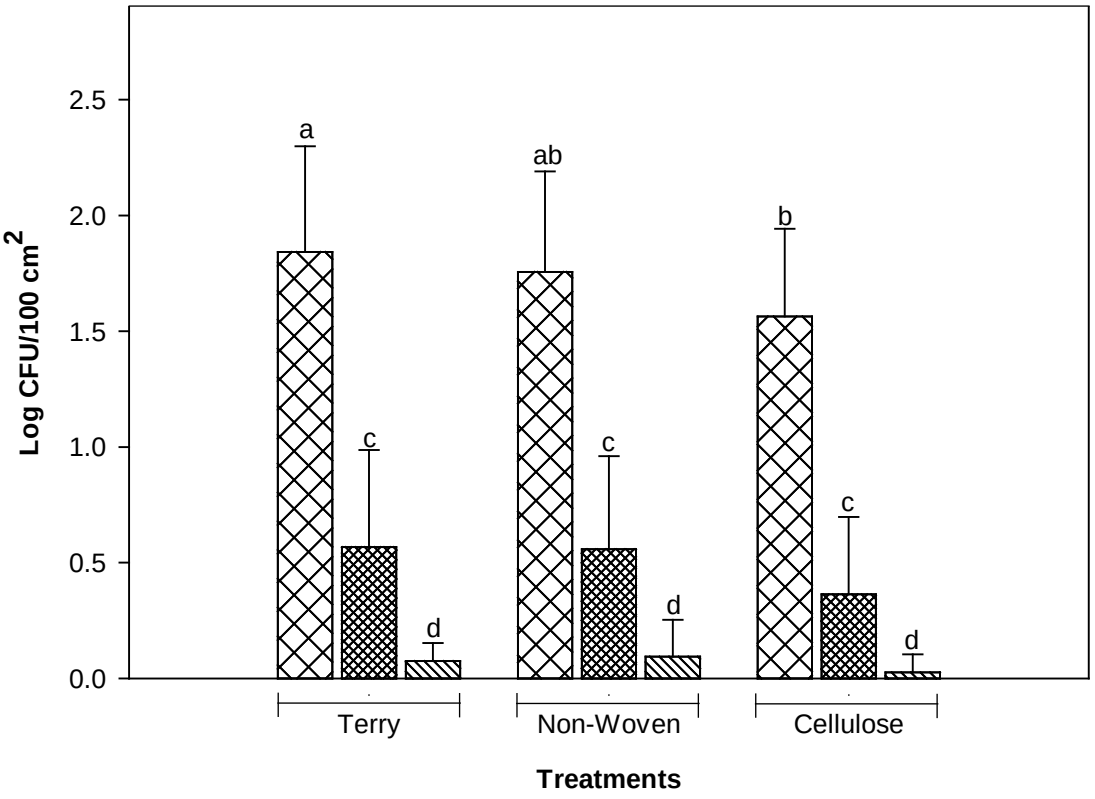

Log Relative Light Units (RLU)/100 cm2
0
1
2
3
a
b
c
a
b
c
a
b
c
Terry
Non-Woven
Cellulose
Treatments

Figure 2


2.5
2.0
1.5
1.0
0.5
0.0
Log CFU/100 cm2
a
c
d
ab
c
d
b
c
d
Terry
Non-Woven
Cellulose
Treatments

CHAPTER IV

LETHALITY OF MOIST HEAT AND SILVER DIHYDROGEN CITRATE DISINFECTANT AGAINST LISTERIA STRAINS INOCULATED ON DELI SLICER COMPONENTS

ABSTRACT

Deli meat slicers have been implicated in the cross-contamination of ready-to-eat (RTE) foods, with *Listeria monocytogenes*, which has resulted in listeriosis outbreaks. We investigated the lethality of silver dihydrogen citrate (SDC) disinfectant, moist heat, and moist heat + SDC disinfectant treatments on a cocktail of *Listeria* strains inoculated on stainless steel and cast aluminum coupons cut from components of a deli meat slicer. Coupons were inoculated, allowed to stand at 4°C for 4 h and subjected to the three treatments. Moist-heat treated coupons were either placed inside the deli meat slicer motor compartment (Internal), which was in turn placed in a deli bread proofer oven, or on an open shelf of the deli bread proofer oven (External), operated on proofer mode at 150 °F (66 °C), for 7 h, with 100 mL deionized water in the proofer water pan. All treatments produced significant ($P < 0.05$) log reductions compared with positive controls. The disinfectant, internal, external moist heat only and both internal and external moist heat + disinfectant treatments caused average log reductions of 4.87 and 5.63; 6.34 and 6.16; 4.49 and 4.87; 6.34 and 6.16 and 6.34 and 6.16 log CFU/cm^2 on SS and AL coupons, respectively. Moist heat only and moist heat + disinfectant treatments at 150 °F (66°C) and at least 20% relative humidity (RH) for 7 h are adequate to attain a 6.34 and 6.16 log reduction of *Listeria* cocktail on SS and AL deli meat slicer components.

INTRODUCTION

Listeria monocytogenes is a Gram positive, non-spore-forming and facultative bacterium which, however, prefers a microaerophilic environment (Ryser and Donnelly 2001). The optimum growth temperature for *Listeria* species ranges between 30 and 37°C; however, this psychrotrophic foodborne pathogen can multiply at temperatures ranging between 1 to 45°C. Its ability to survive and proliferate at refrigeration temperatures is one of the numerous factors that make its control difficult (Liu 2006, Ryser and Donnelly 2001). Among the eight currently known *Listeria* species, only *L. monocytogenes, L. ivanovii,* and *L. seeligeri* have been linked with human pathogenicity (Cocolin 2002). The recently described *L. marthii* and *L. rocourtiae* species have not been associated with human pathogenicity (Graves *et al.* 2010 and Leclercq *et al.* 2010). *L. monocytogenes* is the only species of the genus that has been involved in known food-borne outbreaks of listeriosis (Cocolin *et al*. 2002). All the 13 known *L. monocytogenes* serotypes (*1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e* and *7)* can potentially cause human listeriosis; however, most of the strains isolated from human cases and foods belong to serotype *1/2a, 1/2b* and *4b* (Seeliger and Höhne 1979, Farber and Peterkin 1991).

Most *L. monocytogenes* infections are acquired through ingestion of contaminated food (Montville and Matthews 2008, Schlech *et al*. 1983, Fleming *et al*. 1985, Linnan *et al*. 1988). The infectious dose of the organism is not known; however, it has been reported to be dependent on the susceptibility status of the host, the virulence of the strain, and the type of food product involved (Montville and Matthews *2008,* Lianou and Sofos *2007)*. Levels usually exceeding 100 CFU/g in foods linked to outbreaks have been reported. However, levels as low as less than 0.3 CFU/g have also been reported in some cases such as in the frankfurters incriminated in the 1998 listeriosis outbreak (Montville and Matthews 2008).

The food processing environment has been reported to provide suitable conditions for the growth of *L. monocytogenes*. The organism has been identified in moist areas such as floor drains stagnant water, floors, and processing equipment. It has also been reported to attach on surfaces such as stainless steel,

glass and rubber (Montville and Matthews 2008). Bacteria enter foods as a result of contact with contaminated surfaces (Beresford 2001). Moreover, *L .monocytogenes* has the ability to form biofilms on food contact surfaces rendering them difficult to clean because cells in a biofilm can be significantly more resistant to disinfectants and sanitizers than planktonic cells (Ronner and Wong 1993). Heat processes have been shown to eliminate *L. monocytogenes* from food and food processing equipment and the pathogen gets into processed foods predominantly by post-processing contamination. The organism often colonizes raw materials used in the food preparation establishments making it easy for it to recontaminate previously clean environments (Montville and Matthews 2008).

It has been demonstrated that deli meat slicers pose a potential vehicle of cross-contamination by *L. monocytogenes* during meat processing (Gilbert and Maurer 1968, Lin *et al*. 2006, Vorst *et al.* 2006, Keskinen et al 2008, Sheen and Hwang 2008, Sheen 2008). Several major listeriosis outbreaks in the United States have been traced back to the consumption of delicatessen-sliced meat (CDC 2000, Gottlieb *et al*. 2006). Endrikat *et al.* (2010) reported that 83% of listeriosis deaths linked with deli meats is attributed to retail-sliced products, and that of these estimated deaths 69.8% are attributed to retail-sliced products that do not possess a microbial growth inhibitor such as sodium diacetate and potassium lactate (Lin *et al*. 2006). Of the 500 estimated annual listeriosis fatalities, 242 are believed to be traceable to delicatessen meats. It is estimated that 75% of all luncheon meats sold are being sliced at delicatessens, thus exposing more people to *L. monocytogenes* infection (Vorst *et al.* 2006). Gombas *et al.* (2003) reported a prevalence of 2.7% of *L. monocytogenes* from retail-sliced products compared with 0.4% from prepackaged commercially manufactured and sliced RTE products. Draughon *et al*. (2006) found a similar trend of 1.4% and 0.2% prevalence on retail-sliced and prepackaged products, respectively.

The FDA Food Code (2003) requires that food contact surfaces and equipment used for potentially hazardous foods be cleaned as needed throughout the day at least every 4 hours to prevent the growth of microorganisms on such surfaces. The Code also requires sufficient time for hot water or chemicals to contact utensils or food-contact surfaces to destroy pathogens that may remain on surfaces after cleaning. Disinfectants have been shown to significantly reduce bacterial load from food processing

surfaces (Somers and Wong 2004, Crandall *et al*. 2012); however, organic materials that may remain on cleaned and sanitized food processing equipment such as deli meat slicers, may reduce the effectiveness of such disinfectants resulting in *L. monocytogenes* cells surviving cleaning and sanitization processes. Moist heat has been demonstrated to be more effective in inactivating bacteria than dry heat (Murphy *et al.* 1999, Harrison and Carpenter 1989, Crandall *et al.* 2009). Fujita *et al.* (2010), in their experimental study on the effect of relative humidity on the heat transfer of an evaporating water droplet in air flow, observed a decrease in droplet temperature in a low-relative-humidity condition and an increase in the droplet temperature in a high-relative-humidity condition.

This study investigated the efficacy of moist heat and a combination of moist heat and a disinfectant in inactivating a cocktail of *Listeria* strains inoculated on stainless steel and cast aluminum coupons cut from deli meat slicer components.

MATERIALS AND METHODS

Bacterial cultures and cocktail

We used a cocktail of seven isolates of *Listeria* species including *L. innocua*, *L. monocytogenes* and *L. ivanovii* from the University of Arkansas Center for Food Safety culture collection (Table 1). Stock cultures were kept frozen at -80ºC. The working cultures were prepared by inoculating a loop-full of the individual frozen stock into seven separate tubes containing 10 mL of tryptic soy broth (Bacto, Becton Dickinson Co., Sparks, MD) supplemented with 0.6% yeast extract (TSBYE) and were incubated overnight at 37°C. Each culture was supplemented with 20% glycerol and stored in cryogenic vials (Nalgene, Rochester, NY, USA) at -20°C until use. Working cultures were grown from the -20^{0}C stock by streaking on Modified Oxford (MOX) Agar (MOX; EMD Millipore, Billerica, MA) and incubating at 37°C for 48 h. Single colonies were inoculated in 10 mL of brain heart infusion (BHI) (BBL, Becton, Dickinson, Sparks, Md) plates and incubated at 37°C for 18 to 20 h to yield an approximately 10^9 CFU/ml titer. The inoculation cocktail was prepared by pipetting 1 mL of each of the 7 cultures into a single sterile tube and mixing with a vortex mixer (Thermo Fisher Scientific, Waltham, MA).

Preparation of deli meat slicer coupons

Stainless steel (SS) coupons (1.8 × 2.4 cm) were cut from the blade of a Hobart heavy duty slicer (Hobart Food Equipment, Australia), using a Flow Waterjet Cutting System (Flow International Corporation, Kent, WA). The cast aluminum (AL) blade guard of the slicer was cut into 2 × 2.5 cm coupons, using a Milwaukee Heavy-Duty cold-cutting metal saw (Brookfield, WI) and a Wellsaw metal-cutting band saw (Wells Manufacturing Corporation, Three Rivers, MI). Prior to use in the experiments, the coupons were washed and sanitized using Micro 90 cleaning solution (International Products Corp., Burlington, NJ) prepared according to the manufacturer's instructions and autoclaved for 15 min at 121°C after rinsing in sterile deionized water.

Inoculation of coupons

Coupons were arranged for treatment as shown in Table 2. Inoculation of coupons was done in a biosafety cabinet. Sterilized coupons were separately placed on individual sterile petri dishes and 100 µl of the *Listeria* strain cocktail was pipetted onto the middle of each coupon and carefully spread over the surface area with the pipette tip. The inoculum was allowed to dry and attach on coupons for 4 h at 4°C. We established that recovery of *Listeria* cells was best if coupons were left at 4°C for 3 to 4 h compared with leaving them in the biosafety cabinet or placing them under high humidity conditions when 10^9 CFU/mL of the *Listeria* cocktail was inoculated on the coupons.

Preparation of the deli meat slicer for treatment in bread proofer oven

A deli meat slicer (Model 1812, Hobart Food Equipment, Troy, OH) was partially disassembled as it would during a thorough cleaning procedure, and in order for it to fit into a deli bread proofer oven (Model NHPL-1836, Win-Holt Equipment, Syosset, NY). The slicer was placed in the deli bread proofer oven, plugged in and switched on before and after every thermal treatment to test if it was still normally functioning.

SDC disinfectant and moist heat inactivation of Listeria cells on coupons

Each experiment was run three times with duplicate samples. Following the attachment of *Listeria* cells at 4^0C for 4 h, 16 test coupons (8 AL and 8 SS coupons), prepared for disinfection only and moist heat plus disinfection treatments, were sprayed with silver dihydrogen citrate disinfectant (Pure Bioscience, El Cajon, CA) and allowed to stand for 2 minutes, as indicated by the manufacturer for the treatment of *L. monocytogenes*. Four test coupons (2 AL and 2 SS coupons) prepared for moist heat plus disinfectant treatment were placed in a bread proofer oven shelf (External) together with the same number of coupons prepared for moist heat treatment only (Table 2). Four more moist heat plus disinfectant coupons were placed inside the deli meat slicer motor compartment (MC) (Internal) together with four coupons (2 AL and 2 SS coupons) prepared for moist heat treatment only. Positive controls were inoculated as described, whereas negative controls were inoculated with 100 µL 0.1% buffered peptone water (BPW) only. The initial *Listeria* cocktail inoculum levels were measured for each experiment.

The deli bread proofer oven temperature was set at 150 °F (66 °C) in proofer mode, allowed to run for 7 h, and 100 mL of deionized (DI) water was poured into the proofer oven water pan. Two portable data loggers (HOBO® U12 Temp/RH2; Onset Computer Corporation, Buzzards Bay, MA) were used to log both the temperature and relative humidity. One was placed on an open shelf in the bread proofer oven adjacent to the external test coupons; and the other was placed in the motor compartment (MC) next to the internal test coupons. Prior to moist thermal treatment, the deli slicer MC was reassembled to its normal operational condition. The reason for reassembling the deli meat slicer was to mimic internal harborages in the slicer during thermal treatment (Crandall *et al.* 2011).

Recovery of surviving *Listeria* cells from coupons

Following treatment, all test and control coupons were washed gently by dipping in BPW and transferred into 50 mL sterile centrifuge tubes containing 5 mL and 4.32 mL BPW for AL and SS coupons, respectively. These volumes equal the surface area of the coupons. The tubes were then vigorously agitated on a vortex mixer for two minutes to dislodge most of the cells from the coupons.

Ten-fold serial dilutions were prepared from the resultant suspension, followed by plating, in duplicate, on MOX agar and plates were incubated at 37°C for 48 h.

Statistical analysis

The mean number of surviving colonies per ml was converted to log CFU/cm^2 using the formula;

$$CFU/cm^2 = \frac{(average\ CFU)}{(Total\ surface\ area)\ x\ (Dilution\ factor)}$$

and the log CFU/cm^2 means were calculated. Differences in the mean log CFU/cm^2 were determined using the analysis of variance at the $P < 0.0001$ level of significance in the JMP version 9 (SAS Institutes, Cary, NC).

RESULTS AND DISCUSSION

Subsequent to the attachment of the cocktail of *Listeria* cells on coupons, we were able to recover an average of 6.34 and 6.16 log CFU/cm^2 from positive control stainless steel and cast aluminum coupons, respectively. These levels were comparable with the approximately 7 log CFU/cm^2 recovered by Crandall et al. (2012) from the same types of coupons, even though they used calcium alginate swabs to recover the cells from coupons. All the treatments used in this study were able to cause at least a 4 log reduction. It has been reported that food regulations normally require treatments to reduce food pathogens by at least 5 logs (Crandall *et al*. 2010). The results of this study agree with the findings of other bacterial thermal and sanitizer inactivation studies (Crandall *et al*. 2009, 2012, Murphy *et al*. 1999, 2001, Harrison and Carpenter 1989). Crandall *et al.* (2010) established that moist oven heat was capable of causing a 5 log reduction of *L. innocua* within 3 h at 82 °C. However, in our study we exposed stainless steel and cast aluminum coupons, cut from a deli meat slicer, to moist heat at 150 °F (66 °C) for a longer period of approximately 5 h. Fellows (2009) reported that increasing the temperature decreases thermal death time (z-value) and lowering the temperature increases the z-value. Moreover, the decimal reduction time (D-value) is inversely related to temperature and as such cells die more rapidly at higher temperatures (Fellows 2009). Our study also took into consideration the fact that microbial death rate is directly

proportional to the concentration of microorganisms at any given time. Consequently, this implies that the higher the number of micro-organisms present in a raw material, the longer it takes to reduce the numbers to a specified level (Fellows 2009) particularly if the temperature used is sub-lethal. It is the requirement of the Food Code that the surface temperatures of utensils being sanitized using hot water should reach 71°C (160°F). The actual temperatures should be consistent with the machine manufacturer's operating instructions and within limits specified in the Code. In view of the potential delicate nature of deli meat slicer components such as the electrical components in the motor compartment, we increased the thermal inactivation time, reduced the temperature and ensured controlled relative humidity within the proofer by adding 100 mL DI water in the deli bread proofer oven water pan. Previous studies in our lab compared 40, 100 and 500 mL DI water and concluded that 100 mL DI water would be adequate to provide the much needed humidity while ensuring that the moisture does not remain on the deli meat slicer components to cause damages. Moreover, we ensured that the deli meat slicer operated normally by plugging it on before and after each moist thermal treatment.

External moist heat only and disinfectant only treatments

Coupons placed on the open deli bread proofer oven for moist heat only treatment achieved 4.49 and 4.87 log reduction followed by the sanitizer only treatment with 4.87 and 5.63 logs log reduction (Table 1, Fig 1). The data loggers indicated that the open deli bread proofer oven shelf reached 150 °F (66 °C) after approximately 2 h and that the relative humidity (RH) dropped down to approximately 5% after about 1.5 h, thus exposing coupons to the target temperature for 5 h at approximately 5% RH (Fig 2). Murphy et al. (2001) detected five to three $\log_{10}$ (cfu/g) of *Listeria* in patties after the chicken patties reached a final center temperature of 70 to 80°C, respectively, at a RH ranging between 6 and 10 %.

Crandall *et al*. (2012) demonstrated that sanitizers were able to remove 2 to 3 log CFU/cm^2 of inoculated *Listeria* cocktail attached on stainless steel. Even though SDC disinfectant achieved a higher $\log_{10}$ reduction compared with the sanitizers in the latter study, it could have performed better if the coupons had been cleaned before the application of the disinfectant. Holah *et al*. (1998) have indicated that mechanical cleaning prior to disinfection increases disinfectant efficacy, and in some cases, increases

microbial log reductions. Sanitary food contact surfaces can be attained by removing soil deposits and subsequently applying a sanitizer or disinfectant to reduce the number of residual microorganisms. Pre-cleaning is an important step in a sanitization program as some debris, such as organic matter, may inactivate or lead to decreased effectiveness of disinfectants. Effective regular cleaning can be more important than use of a disinfectant for reducing bacterial concentrations. Cleaning and rinsing alone may achieve a 2 to 3 log reduction (Tebbutt 1984). The brain heart infusion broth in which *Listeria* cells were grown and kept in suspension could have lowered the efficacy of the SDC disinfectant resulting in the comparatively lower average log reduction. The SDC disinfectant manufacturer also indicates the need to clean and rinse surfaces prior to the application of the disinfectant.

Internal moist heat only and both internal and external moist heat + disinfectant treatments

Listeria cells were not detectable on coupons after the moist heat only treatment, for coupons placed in the deli meat slicer MC, and after moist heat + disinfectant treatment both in the MC and the open deli bread proofer shelf. These treatments achieve 6.34 and 6.16 log reduction for on SS and AL coupons, respectively. There was no significant ($P > 0.05$) difference in log reduction between the latter treatments (Fig. 1). The log reduction was significantly ($P < 0.05$) different after moist heat inactivation for coupons placed in the deli meat slicer MC {6.34 logs and 6.16 logs for stainless steel and cast aluminum coupons, respectively} and those placed on the open deli bread proofer oven shelf {4.49 logs (70.7%) and 4.87 logs (79.05%) for stainless steel and cast aluminum coupons, respectively} as shown in Fig. 1 and Table 3.

Data loggers showed that coupons located in the deli meat slicer MC were exposed to 150 °F (66 °C) for only about 2.5 h over the 7 h moist heat treatment period. This suggests that it took approximately 4.5 h for the MC temperature to reach 150 °F (66 °C). These coupons were also exposed to at least 20% RH over the duration of the treatment (Fig 3). Murphy *et al*. (1999) observed a 7 $\log_{10}$ (cfu/g) reduction of *Listeria* cells when chicken breast meat was cooked to 70°C in a water bath. Harrison and Carpenter (1989) were able to achieve a 1.8 to 2.3 log reduction and 4 to 5.6 log reduction after cooking chicken samples at 65.6 to 71.1°C and 73.9, 76.7 and 82.2°C, respectively, even though the heating time(s) was

not indicated in this study. Gibson *et al.* (1999) stated that cleaning chemical effects increase linearly with temperature, and that temperature above the melting points of fat and oils facilitates their removal. They added that high temperatures, however, can increase the persistence of protein soils on surfaces due to the denaturing of the proteins.

CONCLUSIONS

Moist heat and moist heat + disinfectant treatments at 150 °F (66°C) and at least 20% RH for 7 h were adequate to attain a 6.34 and 6.16 log reduction of *Listeria* cocktail on SS and AL deli meat slicer components. Despite the ability of the SDC disinfectant to achieve a 4.87 and 5.63 log reduction on SS and AL coupons, respectively, the study revealed that it may not be safe to rely only on this treatment because it could be affected by the presence of organic materials which may have not been removed during the cleaning and rinsing steps. Of note in this treatment is that the disinfectant was able to cause a more log reduction on SS than AL coupons. This may be attributed to the porosity of AL coupons which may prevent the disinfectant from reaching all the *Listeria* cells. Moist heat treatment at 150 °F (66 °C) and below 20% RH for 7 h may also not be relied upon as it was not able to achieve a 5 log reduction.

A deli slicer is constructed of, among other parts, stainless steel and aluminum components, which may or may not be easily disassembled. These components may become eroded with continued washing and through the use of strong chemical cleaners and disinfectants (CCDR, 1998). The slicer seams, seals, gaskets, eroded metal surfaces and other hard-to-reach areas may accumulate liquids and food debris as well as harbor *L. monocytogenes* and other pathogens if they are not properly maintained, cleaned and disinfected. Sanitizing methods capable of reaching potential harborages that conventional methods may not be able to reach are necessary to minimize *L. monocytogenes* cross-contamination of RTE products by the deli meat slicer in the deli environment (Crandall *et al*. 2011). A special moist heat oven, designed for thermal inactivation of *L. monocytogenes*, or the deli bread proofer oven, operating at the parameters used in this study, may provide additional protection against cross-contamination by this organism.

ACKNOWLEDGMENTS

This research was funded through an American Meat Institute Foundation grant and the United States Department of Agriculture's (USDA) National Integrated Food Safety Initiative (NIFSI, grant number 2010-51110-21004). The Fulbright Program is highly appreciated for presenting an opportunity to conduct this study.

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TABLES

Table 1. *Listeria* strains used in the moist heat thermal inactivation and SDC sanitizer tests

Lab Strain #†	Serotype	Source
*L. innocua*169	M1 (Antibiotic resistance to 50 ppm rifampicin & 250 ppm streptomycin)	Healthy pregnant woman's fecal matter
*L. monocytogenes*187	4b	CDC, cheese associated outbreak
*L. monocytogenes*188	4b	Isolate from trout brine
*L. monocytogenes*189	1/2a	Sliced turkey associated outbreak
*L. monocytogenes*190	1/2a	Human illness outbreak associated
*L. monocytogenes*191	1/2a	Isolated case of human illness
*L. ivanovii*192	-	Smoked fish plant

† Strains were taken from the Center for Food Safety culture collection at the University of Arkansas-Fayetteville.

Table 2. Arrangement of stainless steel (SS) and cast aluminum (AL) coupons used in the moist heat and SDC sanitizer tests

Controls	Coupon Location	Coupon treatment		
		Sanitizer only[a]	Moist heat only[b]	Sanitizer+moist heat[c]
Positive control SS1	MC (INT)	SAN SS1	INT SS1	INT SS1
Positive control SS2		SAN SS2	INT SS2	INT SS2
Positive control AL1		SAN SS1	INT AL1	INT AL1
Positive control AL2		SAN SS2	INT AL2	INT AL2
Negative control SS1	Proofer oven shelf (EXT)	SAN AL1	EXT SS1	EXT SS1
Negative control SS2		SAN AL2	EXT SS2	EXT SS2
Negative control AL1		SAN AL1	EXT AL1	EXT AL1
Negative control AL2		SAN AL2	EXT AL2	EXT AL2

[a] Coupons were removed from 4^0C and placed in the biosafety cabinet where they were sprayed with silver dihydrogen citrate (SDC) and allowed to stand for 5min before recovery of surviving cells.

[b] Coupons were removed from 4^0C and placed directly in the proofer oven for 7 h before recovery of surviving cells.

[c] Coupons were removed from 4^0C and placed in the biosafety cabinet where they were sprayed with SDC and allowed to stand for 5 min before being placed in the proofer oven for the 7 h moist heat treatment.

Table 3. Average CFU log reduction per coupon after each treatment

	Average Log CFU/cm^2 Reduction after Treatment (%)[a]		
Coupon[b]	Sanitizer Only	Moist Heat Only	Moist Heat+Sanitizer
Internal SS[c]	4.47 (70.41)	6.34 (ND)[e]	6.34 (ND)[e]
External SS	5.27 (83.08)	4.49 (70.70)	6.34 (ND)[e]
Internal AL[d]	5.75 (93.36)	6.16 (ND)[e]	6.16 (ND)[e]
External AL	5.51 (89.52)	4.87 (79.05)	6.16 (ND)[e]

[a] Each value is a mean of duplicate samples from 3 experimental runs.

[b] The location of coupons treated with SDC disinfectant only should be ignored because these coupons were not treated in the deli bread proofer oven.

[c] SS, Stainless steel coupon

[d] AL, Aluminum coupon

[e] ND, not detectable

FIGURE LEGEND

Figure 1. Average log CFU/cm^2 of surviving *Listeria* cells inoculated on stainless steel (SS) and aluminum (AL) deli meat slicer coupons and treated by the application of SDC sanitizer only or moist heat only or moist heat + sanitizer treatments. Moist heat treated coupons were either placed inside the deli meat slicer motor compartment (Internal), which was also placed in a deli bread proofer oven, or on an open shelf of the deli bread proofer oven (External) operated on proofer mode at 150 °F (66 °C), for 7 h, with 100 mL deionized water in the proofer water pan. Arrows indicate non-detectable *Listeria* levels for each treatment. Similar letters denote no significant difference ($P < 0.05$) in mean CFU/cm^2.

Figure 2. Data logger readout showing that the proofer oven (External) temperature rose to approximately 150°F (66°C) after about 2 h and that the relative humidity (RH) dropped down to less than 5% after about 1.5 h, thus exposing coupons to the target temperature for 5 h at 5% RH.

Figure 3. Data logger readout showing that the deli meat slicer motor MC (Internal) temperature rise to approximately 150°F (66°C) after about 4.5 h and the relative humidity (RH) steadily dropping down to 20% over the duration of 7 h, thus exposing coupons to the target temperature for approximately 2.5 h at or above 20% RH.

FIGURES

FIGURE 1

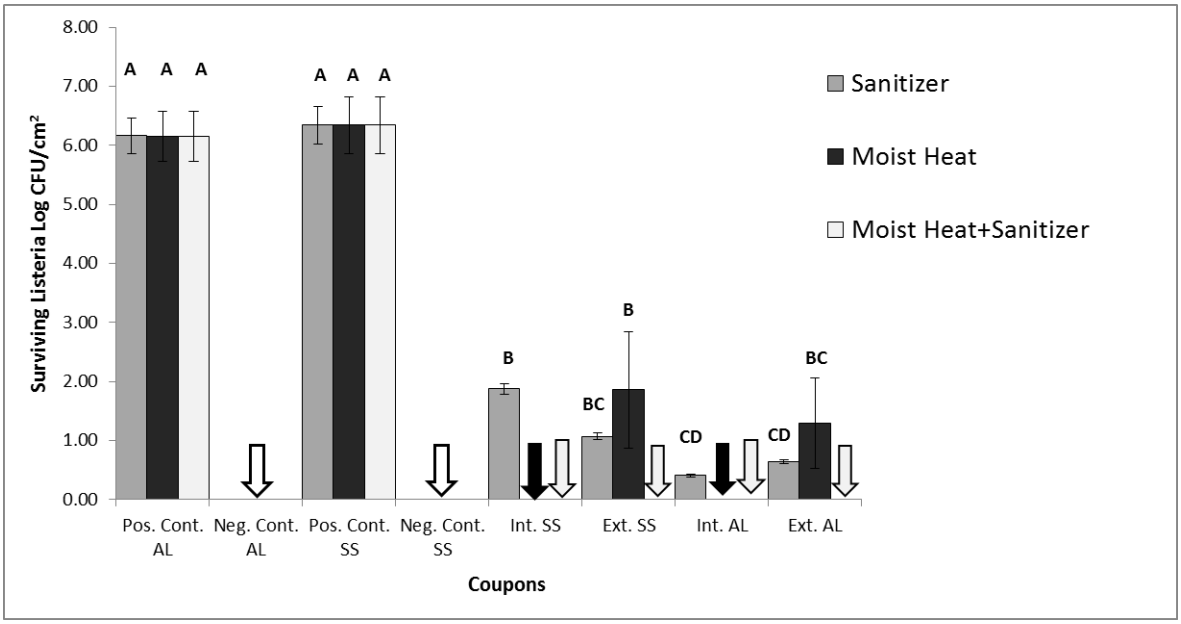


FIGURE 2

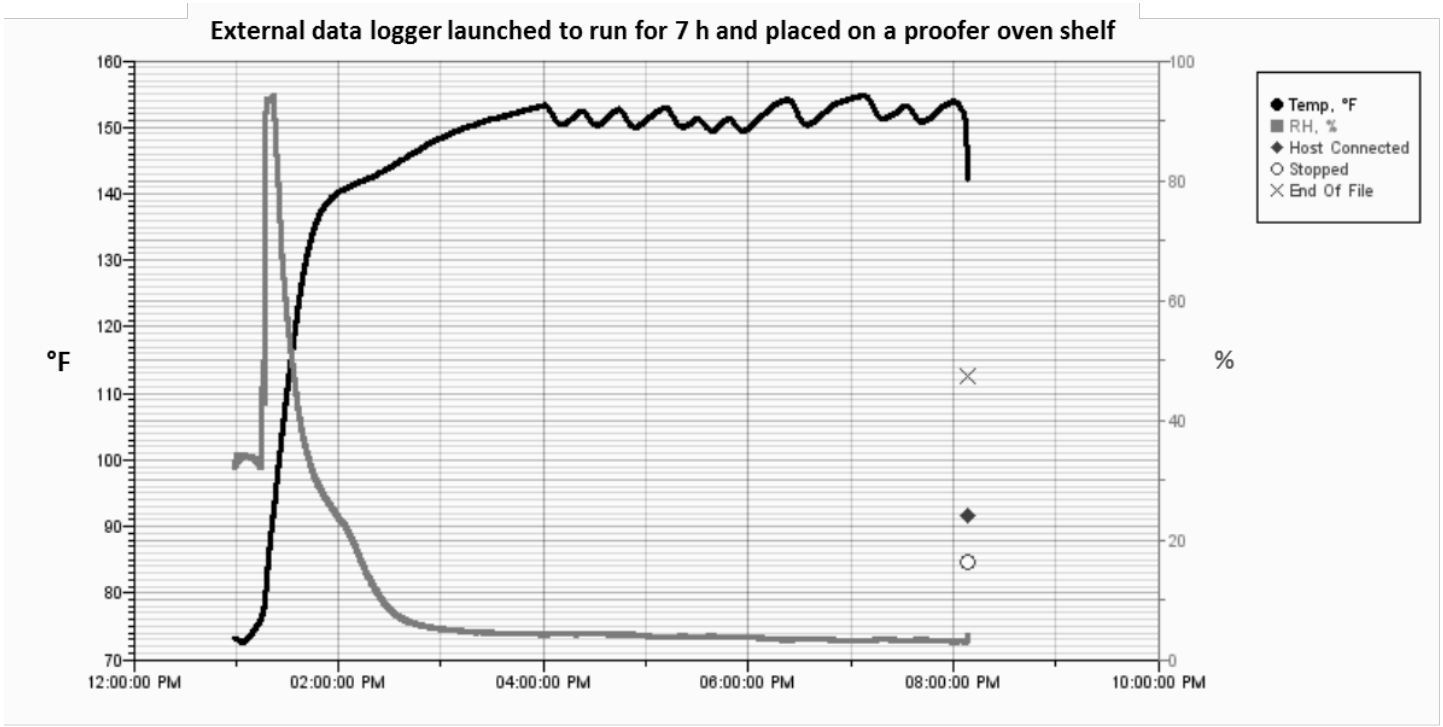
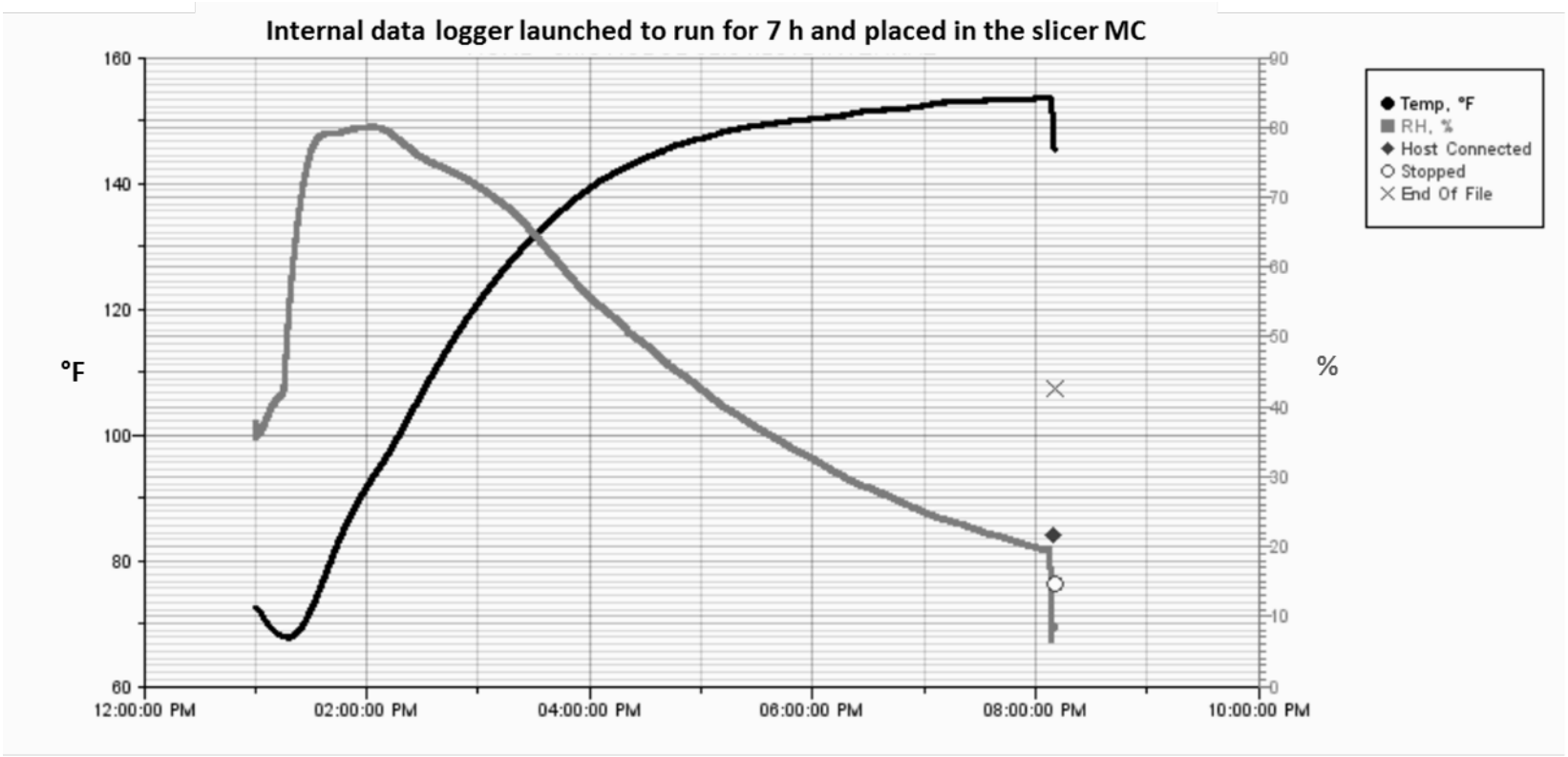

External data logger launched to run for 7 h and placed on a proofer oven shelf
°F
%
160
150
140
130
120
110
100
90
80
70
100
80
60
40
20
0
12:00:00 PM
02:00:00 PM
04:00:00 PM
06:00:00 PM
08:00:00 PM
10:00:00 PM
Temp, °F
RH, %
Host Connected
Stopped
End Of File

FIGURE 3


Internal data logger launched to run for 7 h and placed in the slicer MC
°F
%
160
140
120
100
80
60
90
60
70
60
50
40
30
20
10
0
12:00:00 PM
02:00:00 PM
04:00:00 PM
06:00:00 PM
08:00:00 PM
10:00:00 PM
Temp, °F
RH, %
Host Connected
Stopped
End Of File

CHAPTER V

VIABLE-BUT-NON-CULTURABLE STATE OF LISTERIA STRAINS SUBJECTED TO SUB-LETHAL MOIST HEAT AND SILVER DIHYDROGEN CITRATE DISINFECTANT STRESSES

ABSTRACT

Listeria monocytogenes has been reported as one of the few gram-positive bacteria capable of converting to the viable-but-non-culturable (VBNC) state when exposed to stresses such as sub-lethal temperatures. The purpose of this study was to determine if *Listeria* strains convert to the VBNC state when subjected to sub-lethal moist heat and silver dihydrogen citrate disinfectant. A log-phase cocktail of *Listeria* strains was inoculated on stainless steel (SS) and cast aluminum (AL) coupons cut from components of a deli meat slicer, allowed to stand for 4 h at 4°C and then subjected to moist heat, silver dihydrogen citrate (SDC) disinfectant, and moist heat + SDC disinfectant treatments. A deli meat slicer was placed inside a bread proofer oven and inoculated test coupons were placed inside the motor compartment and outside of the slicer on the proofer oven shelf. The proofer oven was operated on proofer mode at 150 °F (66 °C), for 7 h, with 100 mL deionized water poured on the proofer water pan. Fluorescence microscopy, using the Live/Dead BacLight™ Bacterial Viability kit, and selective nutrient plate count were used to test bacterial viability and growth on treated coupons. The plate count detected 1.28 and 0.65 log CFU/cm^2 on both external moist-heat treated SS and AL coupons, respectively. However, the Live/Dead BacLight™ Bacterial Viability test detected 2.73 and 3.68% on internal moist-heat treated SS and external moist-heat treated AL coupons, but nothing was detected on external moist-heat treated SS coupons. Despite the effectiveness of the moist heat treatments, demonstrated by the low % live cells after treatment, there was an indication that these treatments may induce the VBNC state in *Listeria* strains. The conversion of *L. monocytogenes* into the VBNC state is of great concern for microbial food safety.

INTRODUCTION

Listeria monocytogenes has continued to cause listeriosis outbreaks worldwide despite stringent measures put in place for its control in the USA and in Europe (Shank *et al*., 1996, Carrasco *et al*. 2007). Liseriosis is a rare disease: however, it has a very high fatality rate, especially in immuno-compromised and pregnant women (Mead *et al*., 1999). *L. monocytogenes* has been reported to cause infection by several transmission routes including consumption of contaminated foods (Schlech *et al.*1983, Fleming *et al.*1985, Linnan *et al.*. 1988), mother to fetus *in utero* transmission, direct infection of the fetus at the time of birth, or by direct contact with the organism, which causes lesions on the skin (McLauchlin 1990). Most *L. monocytogenes* infections are, however, acquired through ingestion of contaminated food (Montville and Matthews 2008).

Several studies have revealed that contamination of foods by *L. monocytogenes* can occur at any point in the food chain, including farms, in food processing plants, in retail establishments and in the home (Lappi 2004, Nightingale 2005, Saunders 2006). Petran and Zottola (1989) have noted that despite the fact that *Listeria* can be easily inactivated by cooking and pasteurization, exposure of food in the processing plant environment after cooking but before packaging may result to cross-contamination. For this reason *L. monocytogenes* remains an important pathogen in ready-to-eat foods such as delicatessen meats (Tompkin 2002). It has been demonstrated that *L. monocytogenes* has the ability to colonize, proliferate, and thrive in the food processing environment, including food processing equipment, for long periods (Lappi *et al.*, 2004, Kabuki *et al..*, 2004). *Listeria* can easily adapt in the environment and can even survive freezing, surface dehydration and spray chilling conditions, but it can be easily killed with proper cooking (Seeliger and Jones 1986, Junttila *et al.*1988).

It has been established that when bacteria are exposed to stressful environmental conditions such as nutrient depletion, temperatures outside the optimal range of growth, elevated osmotic concentrations such as seawater, oxygen concentration, or exposure to white light they transform to a viable-but-non-culturable (VBNC) state (Xu *et al.* 1982, Oliver 2005, Rice *et al.* 2000). The VBNC state of bacteria has

been defined as the inability of bacteria to be cultured on media which normally supports its growth, and yet they still exhibit indicators of metabolic activity (Oliver 2000). Bacteria in this state have been reported to be capable of being revived to a metabolically active state when the conditions permit (Oliver 2000). This "non-recoverable" stage of existence adopted by bacteria in their normal culture media was first described by Xu *et al.* (1982) in their study involving *Escherichia coli* and *Vibrio cholerae* in an aquatic environment.

Sub-lethal stress is reported to modify the metabolic activities of the cells. It can result in microbial "injury" and can be expressed as either retarded growth or complete prevention of growth (Donnelly 2002). Lethal or severe stress causes irreversible damage to the microbial cells. When microorganisms are exposed to sub-lethal stress, they resort to "stress adaptation" (Lou and Yousef 1997), which is the condition in which an organism becomes more resistant to subsequent levels of the same stress or at times to a different stress (Hill *et al*. 2002). Microbial stress adaptation is very important in food processing where pathogens may adapt to microbial "hurdles" or stressors and can survive under conditions in which they would have been otherwise inactivated (Beales 2004).

The optimal growth temperature for *L. monocytogenes* is between 30°C and 37°C, and any temperature above this optimal range is expected to exert a stress (Petran and Zottola 1989). When microbial cells are exposed to temperatures above optimal growth temperatures even for short periods of time, they tend to synthesize heat shock proteins (Lindquist 1986; Knabel et al. 1990).

Some gram-positive bacteria including *L. monocytogenes* have been reported as having the potential to enter the VBNC state (Byrd *et al.* 1991, Besnard *et al*. 2000, Li *et al.* 2003). A 7-fold increase in thermo-tolerance of *L. monocytogenes* has been observed when the cells were exposed to 45°C for 3 h (Pagàn *et al*. 1997). Gunasekera *et al.* (2002) demonstrated that a large number of VBNC *Escherichia coli* and *Pseudomonas putida* cells, in pasteurized milk, were metabolically active after pasteurization for 30 min at 63.5°C even though they were not able to form colonies. The fact that VBNC cells cannot be detected by traditional culturing methods yet they are capable of resuscitation and growth into the

culturable state under favorable conditions makes them potential threats to public health (Byrd *et al.* 1991, Besnard *et al.* 2000, Oliver 2010).

The Live/Dead BacLight Bacterial Viability Kit has been widely used for the characterization of bacterial viability in many fields including food safety and environmental monitoring since it was released more than a decade ago (Boulos *et al*. 1999). The kit consists of two stains; propidium iodide (PI) and SYTO9, which both have the affinity for nucleic acids (Stocks 2004). The green fluorescing SYTO9 is an intercalating stain that is able to penetrate all cell membranes and bind to nucleic acid resulting to the green fluorescence, and is usially used for assessing total cell counts (Berney *et al*. 2007). The red fluorescing PI is an intercalating stain that, due to its large and highly charged molecules, can enter only cells with injured cytoplasmic membranes (Berney *et al*. 2007). The emission properties of the stain mixture bound to DNA are reported to change because the mechanism is a combination of displacement of SYTO9 by PI and quenching of SYTO9 emissions by fluorescence resonance energy transfer (FRET) (Stocks 2004, Berney *et al*. 2007). This suggests that PI has a stronger affinity for nucleic acid and that, when the two stains are present within a cell, SYTO9 will be displaced from nucleic acid and the cells will fluoresce in red (Stocks 2004). Confusing results can occur if the relative intensities of the stains or the concentration of PI in relation to nucleic acid are not properly accounted for (Stocks 2004).

We subjected a cocktail of *Listeria* strains to moist heat at 150 °F (66 °C) for 7 h and to a combination of moist heat (at the same temperature) and silver dihydrogen citrate disinfectant. The purpose of this study was to determine if *Listeria* cells subjected to these levels of stresses converted into the VBNC state.

MATERIALS AND METHODS

Bacterial strains and cocktail preparation

The *Listeria* strains used in this study included three isolates of *L. monocytogenes* serotype 1/2a, two isolates of serotype 4b, one isolate of *L. innocua* M1, and one isolate of *L. ivanovii*. All cultures were

obtained from the University of Arkansas Center for Food Safety culture collection. Cultures were prepared from -80ºC stock by inoculating a loop-full of the individual frozen stock into seven separate tubes containing 10 mL tryptic soy broth (Bacto, Becton Dickinson Co., Sparks, MD) supplemented with 0.6% yeast extract (TSB-YE). The tubes were incubated at 37°C for 18 to 20 h. Each culture was supplemented with 20% glycerol and stored in cryogenic vials (Nalgene, Rochester, NY, USA) at -20°C until use. Cultures were grown from the -20 °C stock by streaking on Modified Oxford (MOX) Agar (MOX; EMD Millipore, Billerica, MA) and incubated at 37°C for 48 h. Single colonies were cultured on 10 mL brain heart infusion (BHI) (BBL, Becton, Dickinson, Sparks, Md) overnight, plated on MOX plates and incubated at 37°C for 48 h to yield an approximately 10^9 CFU/ml titer.

Overnight cultures of each *Listeria* strain were inoculated in fresh BHI and incubated at 37 °C with agitation at 100 rpm followed by an hourly spectrophotometer (Beckman DU530, Beckman Coulter Inc, Brea, CA, USA) measurement of the absorbance of each strain culture until an optical density of 600 nm (OD_{600}) for each culture was reached that represented the log phase (~ 10^9 CFU/mL) of the culture (Fig. 1, 2, 3). The inoculation cocktail was then prepared by pipetting 1 mL of each of the 7 log-phase cultures into a single sterile tube and mixed with a vortex mixer (Thermo Fisher Scientific, Waltham, MA). The cocktail was pelleted at 4000 x g for 7 min to harvest log phase cells and then re-suspended in 7 ml 0.85% NaCl.

Preparation of live/dead *Listeria* cells

Live cells were prepared by adding 1 ml of the cocktail in 20 ml of 0.85% NaCl and incubated for 1 h at room temperature while mixing every 15 min. However, dead cells were prepared by adding 1 ml of the cocktail in 20 ml isopropanol and incubated for 1 h at room temperature with mixing every 15 min. Both suspensions were washed twice in 20 ml 0.85% NaCl by centrifugation at 4000 x g for 7 min and separately re-suspended in 10 ml 0.85% NaCl.

Preparation of live/dead standard curves

Standard curves were prepared using live and dead cells as per the viability test kit manufacturer's recommendations. Briefly, different volumes of live and dead-cell suspensions were prepared to achieve five proportions of live:dead cells for the fluorescence microplate reader (BioTek, Winooski, VT). The live (green)/dead (red) ratio was plotted against percentage of live cells in the Listeria cocktail suspension. The percentage of live cells after each experimental treatment was then estimated based on these standard curves as described below.

Preparation and inoculation of coupons

Stainless steel (1.8 × 2.4 cm) and cast aluminum (2 × 2.5 cm) coupons were, respectively, cut from the blade of a Hobart heavy duty slicer (Hobart Food Equipment, Australia) using a Flow Waterjet Cutting System (Flow International Corporation, Kent, WA), and from the blade guard of the slicer using a Milwaukee Heavy-Duty cold-cutting metal saw (Brookfield, WI) and a Wellsaw metal-cutting band saw (Wells Manufacturing Corporation, Three Rivers, MI). Before use in each experiment, the coupons were washed and disinfected using Micro 90 cleaning solution (International Products Corp., Burlington, NJ) according to the manufacturer's instructions, rinsed in sterile deionized water, and autoclaved for 15 min at 121°C. The *Listeria* strains cocktail (100 µL) was pipetted onto the middle of the sterilized coupons, in a biosafety cabinet, and carefully spread over the surface area with the pipette tip. The inoculum was allowed to dry and attach on coupons for 4 h at 4^0C.

A deli meat slicer (Model 1812, Hobart Food Equipment, Troy, OH) was partially disassembled as it would during a thorough cleaning procedure, and in order for it to fit into a deli bread proofer oven (Model NHPL-1836, Win-Holt Equipment, Syosset, NY). The slicer was placed in the deli bread proofer oven, plugged in and switched on before and after every thermal treatment to test if it was still normally functioning.

Treatment of *Listeria* cells on coupons

Inoculated coupons prepared for disinfection only and moist heat plus disinfection treatments, were sprayed with silver dihydrogen citrate disinfectant (Pure Bioscience, El Cajon, CA) and allowed to stand for 2 minutes, as indicated by the manufacturer for the treatment of *L. monocytogenes*. Coupons prepared for moist heat plus disinfectant and moist heat only treatments were placed on a bread proofer oven shelf (External) (Model NHPL-1836, Win-Holt Equipment, Syosset, NY). The same number of coupons was also placed inside the deli meat slicer motor compartment (MC) (Internal). Positive controls were inoculated as described and negative controls were inoculated with 100 µL of 0.85% NaCl only. The initial *Listeria* cocktail inoculum levels were measured for each experiment.

The deli bread proofer oven temperature was set at 150 °F (66 °C) on proofer mode, allowed to run for 7 h, and 100 mL of deionized (DI) water was poured into the proofer oven water pan. Two portable data loggers (HOBO® U12 Temp/RH2; Onset Computer Corporation, Buzzards Bay, MA) were used to log both the temperature and relative humidity. One was placed on the open shelf in the bread proofer oven adjacent to the external test coupons; and the other was placed in the MC next to the internal test coupons. Prior to moist thermal treatment, the deli slicer MC was reassembled to its normal operational condition in order to simulator internal harborages in the slicer during thermal treatment.

Recovery of surviving *Listeria* cells from coupons

Test and control coupons were washed gently by dipping in 0.85% NaCl and then transferred into 50 mL sterile centrifuge tubes containing 5 mL and 4.32 mL 0.85% NaCl for AL and SS coupons, respectively. These volumes equal the surface area of the coupons. The tubes were then vigorously agitated on a vortex mixer for two minutes to dislodge most of the cells from the coupons. Ten-fold serial dilutions were prepared from the resultant suspension, followed by plating, in duplicate, on MOX agar and plates were incubated at 37^{o}C for 48 h.

Live/Dead BacLight™ viability Assay

The LIVE/DEAD BacLight™ Bacterial Viability Kit (Molecular Probes, Invitrogen, CA) was used to determine the viability of the cells after treatment. The manufacturer's protocols were used for the staining procedure. Briefly, for the three treatments, 18 µL of each one of the two stains, SYTOÒ 9 (component A) and propidium iodide (component B) were mixed in a microfuge tube and the entire 36 µL mixture was added to 6 mL of filter sterilized dH_2O in a glass test tube, and mixed well. The stain was prepared such that it would be adequate for the staining of all 100 µL suspensions from the treated coupons. The recovered *Listeria* cell suspensions (100 µL) from each coupon were pipetted into a 96-well flat-bottom microplate. The staining solution (100 µL) was added to each well and mixed thoroughly by pipetting up and down several times. The 96-well flat-bottom microplate containing the samples was incubated in the dark for 15 min at room temperature. After incubation, the samples were measured at two wavelengths (E_{528} and E_{645}) using a fluorescence microplate reader. The excitation wavelength used was 485 nm. The green fluorescence intensity (528 nm, F em1) and red fluorescence intensity (645 nm, F em2) were measured, and the green/red fluorescence ratio (G/R) (E_{528}/E_{645}) was calculated for each experimental sample based on the following formula:

$$Ratio\ G/R = \frac{\text{Fcell, em1}}{\text{Fcell, em2}}$$

RESULTS AND DISCUSSION

In this study we showed that moist heat and a combination of moist heat and the SDC disinfectant at 150 °F (66 °C) for 7 h with the relative humidity (RH) above 10% was effective in inactivating *Listeria* cells as shown by the count plates results and that these treatments may induce the VBNC state in *Listeria* strains as indicated by the Live/Dead BacLight™ bacterial viability test. Table 1 shows a comparison of the plate count results in CFU/cm^2 and the corresponding percent live cells detected by the microplate reader on each test and control coupon using the Live/Dead BacLight™ bacterial viability test.

Moist heat only treatment (internal)

The internal moist heat only treatment achieved non-detectable levels of *Listeria* cells on the nutrient agar plates; however, the Live/Dead BacLight™ bacterial viability test detected a minimal proportion of 2.73% live cells on AL coupons. The temperature (66 °C) used in this experiment is believed to be sub-lethal, but effective in the inactivation of *Listeria* strains when combined with more that 10% relative humidity (RH) as observed in our previous study on the lethality of moist heat on *Listeria* strains. The motor compartment (internal) of the deli meat slicer machine has displayed the ability to maintain RH at above 10% over the 7 h heat treatment of the deli meat slicer in a deli bread proofer oven. This temperature-RH combination has demonstrated effectiveness in inactivating *Listeria* cells after 7 h by consistently achieving non-detectable levels of *Listeria* cells on nutrient agar plates in our previous study. Several studies have established the effectiveness of moist heat in inactivating bacteria (Murphy et al. 1999, Harrison and Carpenter 1989, Crandall et al. 2009). Fujita et al. (2010) has demonstrated the effect of higher RH on heat transfer compared with low-relative-humidity conditions. The detection of a minimal percent live *Listeria* cells on coupons by the bacterial viability test after the moist heat only treatment, but no growth on agar plates, suggests that an insignificant number of cells converted to the VBNC state. It has to be pointed out, however, that the limited data set (from three experimental runs) presented in this study suggests a need for caution in making far reaching conclusions. Further investigation is still required to make valid conclusions.

Moist heat only treatment (external)

Conversely, we demonstrated that when the RH drops below 10%, even if the same time-temperature combination is maintained, inactivation of *Listeria* cells is not adequately achieved (4.45 and 2.65 $\log_{10}$ reduction for SS and AL coupons, respectively). The selective nutrient agar plate count detected an average of 1.28 and 0.65 log CFU/cm^2 on SS and AL coupons, respectively, but the Live/Dead BacLight™ bacterial viability test did not detect any live cells on stainless steel coupons while 3.68% (of 103 counted *Listeria* cells) were detected on cast aluminum coupons after the external moist

heat only treatment (Table 1, Fig. 2). We established in our lab that relative humidity (RH) drops within 1.5 h to less than 10% in the deli bread proofer oven, resulting in less effective time-temperature-RH combination required for inactivating *Listeria* cells. Crandall *et al*. (2010) established that dry oven heat was not capable of causing a 5 log reduction of *L. innocua* within 15 h at 80 °C. Murphy *et al.* (2001) detected five to three $\log_{10}$ (cfu/g) of *Listeria* in patties after the chicken patties reached a final center temperature of 70 to 80°C in a concection oven with 6 to 10% RH. However, in a different study Murphy *et al.* (1999) observed a 7 $\log_{10}$ (cfu/g) reduction for *Listeria* when chicken breast meat was cooked to 70°C in a water bath. These two studies highlighted the importance of moist heat in in heat transfer. However, the Live/Dead BacLight™ bacterial viability kit did not detect any live cells on SS coupons whereas the plate count did (Table 1). The kit manufacturer's manual indicates that the results yielded by the Live/Dead BacLight™ bacterial viability test, with log phase or exponential growing cells, typically correlate with growth assays in liquid or solid media. We therefore expected our results from both the selective nutrient agar plate counts and the Live/Dead BacLight™ bacterial viability test to correlate. Barney et al. (2007) have, however, established that staining of bacterial cells with SYTO9 and propidium iodide does not always produce distinct "Live" and "dead" cells and that an intermediate state has been observed. The authors further report that this phenomenon may complicate interpretation of results and may further confound decisions, for example, on the effectiveness of sanitation methods. The Live/Dead BacLight™ bacterial viability test principle is based on the assumption that injured cells are dead (red labeled), however, it may not be appropriate to always assume that intact cells (green labeled) are active or viable (Joux and Lebaron 2000). Further investigation of the findings of this study is required.

Disinfectant only treatment

We further showed that SDC disinfectant does not induce the VBNC state in *Listeria* strains inoculated on clean sterile stainless steel and cast aluminum coupons. The disinfectant achieved non-detectable levels of *Listeria* cells on both the nutrient agar plates and the Live/Dead BacLight™ bacterial viability test for both the SS and AL coupons. The SDC disinfectant is a ready to use disinfectant which

has broad spectrum and residual disinfectant characteristics against bacteria, fungi, and viruses. It is a solution of silver ions stabilized in citric acid and believed to damage an organism's DNA and protein structure by lysing the cell membrane which results to the cell death (Purebio 2012). The manufacturer cautions that, for the disinfectant to be effective, surfaces must be thoroughly washed prior to its application. In this experiment washed *Listeria* cells were inoculated on clean sterile coupons, which might have enhanced the effectiveness of the disinfectant.

Moist heat + disinfectant treatments

The moist heat plus disinfectant treatment combination both within the deli meat slice MC (internal) and the deli bread proofer shelve (external) achieved non-detectable levels of *Listeria* cells on MOX nutrient agar plates but the viability test showed 3.25% live cells on AL coupons. Previous experiments in our lab have exhibited consistent non-detectable levels of *Listeria* cells on MOX selective nutrient agar plates after exposure to both treatments. Disinfectants have been shown to significantly reduce bacterial load from food processing surfaces (Somers and Wong 2004, Crandall *et al*. 2012). Gibson *et al* (1999) stated that cleaning chemical effects increase linearly with temperature, and that temperature above the melting points of fat and oils facilitates their removal. For this reason we expected these treatments to be more effective than the other treatments discussed above. The relatively low percent live cells detected after treatment requires further investigation and may be an indication of experimental error. However, the 3.25% detected live cells in the viability test against non-detectable levels in nutrient agar plates may suggest the conversion of *Listeria* cells to the VBNC state. VBNC cells display low metabolic activity and are not culturable on nutrient rich media (Li *et al*. 2003) as demonstrated in this study. The exhibition of lack of culturability is used, among other criteria, as a criterion to determine the presence of VBNC cells (Li *et al*. 2003).

Recovery of cells from positive control coupons

An average of 5.73 and 3.30 log CFU/cm^2 was recovered from SS and AL coupons, respectively (Fig. 2, Table 1). The viability test showed 99.92 and 6.62% live cells on SS and AL coupons,

respectively. It is worth noting that the recovered average log CFU/cm^2 in this study is lower for both SS and AL coupons than in the previous deli slicer study. The inoculum level in the current study was at an average of 9.6 CFU/ml, which is equivalent to that of the previous study, but the recovered average concentration in the previous study was above 6 log CFU/cm^2.This is an indication that we must have lost cells during the holding period of 4 h at 4°C. Washing the cells and letting them stand for 4hrs at 4°C exposes them to harsh and stressful conditions which may have resulted to a further reduction in live cells (Li et al. 2003). Nutrient depletion, among other stresses can result to cells converting to the VBNC state (Oliver 2005). Lindback *et al*. (2010) induced the VBNC state in *L. monocytogenes* Scott A by starving the pathogen in microcosm water at 4°C for 12 weeks. Donnelly (2002) reported that sub-lethal stress can result in microbial "injury" which can be expressed as either retarded growth or complete prevention of growth. Li *et al*. (2003) found that refrigeration temperature (4°C) and carbon dioxide, normally used in preservation and modified atmosphere processes, respectively, do not induce the VBNC state in *L. monocytogenes*. This could be explained by the characteristic psychrotrophic and facultative nature of *L. monocytogenes*, respectively (Ryser and Donnelly 2001).

CONCLUSION

The results of this study are still preliminary, and more experiments still need to be carried out to ensure more accurate results. Moreover, optimization of the cell recovery protocol is necessary. The cocktail, in BHI broth, could be inoculated on the coupons before the cell washing step to ensure that the cells have at least adequate nutrients during the 4 h attachment period at the refrigeration temperature Lindback *et al*. (2010). Cells from control and test coupons could then be washed after attachment and treatments, respectively. The suspension of cells in the BHI broth would mimic real life conditions in the deli environment where cells are attached to surfaces contaminated with organic material.

Another step that needs optimization is the development of the standard curve used to estimate the percent live *Listeria* cells after each experimental treatment (Fig.1). In this study only three replications of the five different proportions of live:dead cells were made for fluorescence intensity

measurement in the fluorescence microplate reader. The resultant standard curves were not linear (R^2 < 0.99), however, in this study we used the best standard curve (R^2 = 0.971) generated (Fig. 1). Swarts *et al*. (1998) obtained high correlations for flow cytometric detection of defined proportions of live or dead *L. monocytogenes* cells in mixtures between 10 and 100% of dead (r^2 = 0.97) or live (r^2 = 0.99) cells. Using up to ten replications of the live:dead cells proportions could improve the results of the viability test as demonstrated by the manufacturer in the BacLight™ bacterial viability kit manual (http://probes.invitrogen.com/media/pis/mp34856.pdf) where each point in the standard curve represented the mean of ten measurements. Repeating the fluorescence measurements of the five live:dead proportions of the *Listeria* cocktail sample would also further assist in determining the appropriate proportions of the two fluorescence dyes for this experiment.

In determining the optical density of the each strain at 600 nm (OD_{600}), we used an OD that represented the exponential stage of each strain. Berney *et al.* (2007) have noted that bacterial staining properties are dependent on the physiological condition of the bacteria. It has been reported that log-phase cells stained with the two fluorescence dyes would exhibit higher green fluorescence intensity levels than stationary-phase cells due to wall toughening of stationary cells. Cells in the stationary phase are reported to be more resistant to stresses like mild heat or sunlight than are cells from the log phase (Berney et al. 2006). Moreover, due to increased metabolic activity, log-phase cells have been shown to have higher RNA content which may lead to enhanced fluorescence intensity and thus influence the staining properties of the cells (Berney et al. 2007). In the current study, the use of the log-phase cells may have increased the intensity of the green fluorescence, thus giving the impression that there were more “live” cells (green fluorescence) observed in the samples than “dead” cells (red fluorescence). The use of stationary-phase cells could also be investigated to compare the results of the Live/Dead Baclight™ viability test.

Dipping the treated coupons in an enrichment broth and incubating overnight (18 to 24 h) may give a "yes or no" result regarding the presence/absence of live cells on treated coupons, but it may not

indicate if some of the cells have entered the VBNC state. However, this test may be used to further confirm if there are any viable and culturable cells remaining on the treated coupons. The VBNC state is of public health importance because cells may be resuscitated once conditions become favorable.

Despite the effectiveness of the treatments, as demonstrated by the low percent live cells after treatment, there is some indication of live cells remaining on the coupons after exposure to most of the treatment methods. The absence of growth on nutrient media and detection of live cells by the viability test may suggest that some *Listeria* cells converted to the VBNC state after exposure to the treatments. However, the proportion of detected live cells was less than 4% for all the treatments as indicated in Table 1, which might suggest the effectiveness of these treatments.

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TABLES

Table 1. Percent viability of *Listeria* cells and log CFU after treatment

			Live/Dead BacLight™ viability test
		Plate Count[†]	Fluorescence Microplate Reader[†]
Treatment	Coupon	Log CFU/cm^2	% Live *Listeria* Cells
Disinfectant	Disinf SS[a]	ND	ND
	Disinf SS	ND	ND
	Disinf AL[b]	ND	ND
	Disinf AL	ND	ND
Moist heat only	Internal SS	ND	ND
	External SS	1.28	ND
	Internal AL	ND	2.73
	External AL	0.65	3.68
Moist heat + sanitizer	Internal SS	ND	ND
	External SS	ND	ND
	Internal AL	ND	3.25
	External AL	ND	ND
Positive Control	SS	5.73	99.92
	AL	3.30	6.62
Negative Control	SS	ND	ND
	AL	ND	ND

[†] ND, Not detectable
[a] SS, Stainless steel
[b] AL, cast aluminum

FIGURE LEGENDS

Figure 1. Growth curve of 7 *Listeria* strains, used to make the *Listeria* cocktail in the first experimental run, prepared by plotting the optical density of each strain against time in hours

Figure 2. Growth curve of 7 *Listeria* strains, used to make the *Listeria* cocktail in the second experimental run, prepared by plotting the optical density of each strain against time in hours

Figure 3. Growth curve of 7 *Listeria* strains, used to make the *Listeria* cocktail in the third experimental run, prepared by plotting the optical density of each strain against time in hours

Figure 4. Standard curve showing correlation between E_{528} (green)/E_{645} (red) ratio and percent viability OF *Listeria* cells using the Live/Dead BacLight™ Bacterial Viability Test

Figure 5. Average log CFU/cm^2 of surviving *Listeria* cells inoculated on stainless steel (SS) and aluminum (AL) deli meat slicer coupons subjected to SDC disinfectant only or moist heat only or moist heat + sanitizer treatments. Arrows indicate non-detectable *Listeria* levels for each treatment.

FIGURES

Figure 1

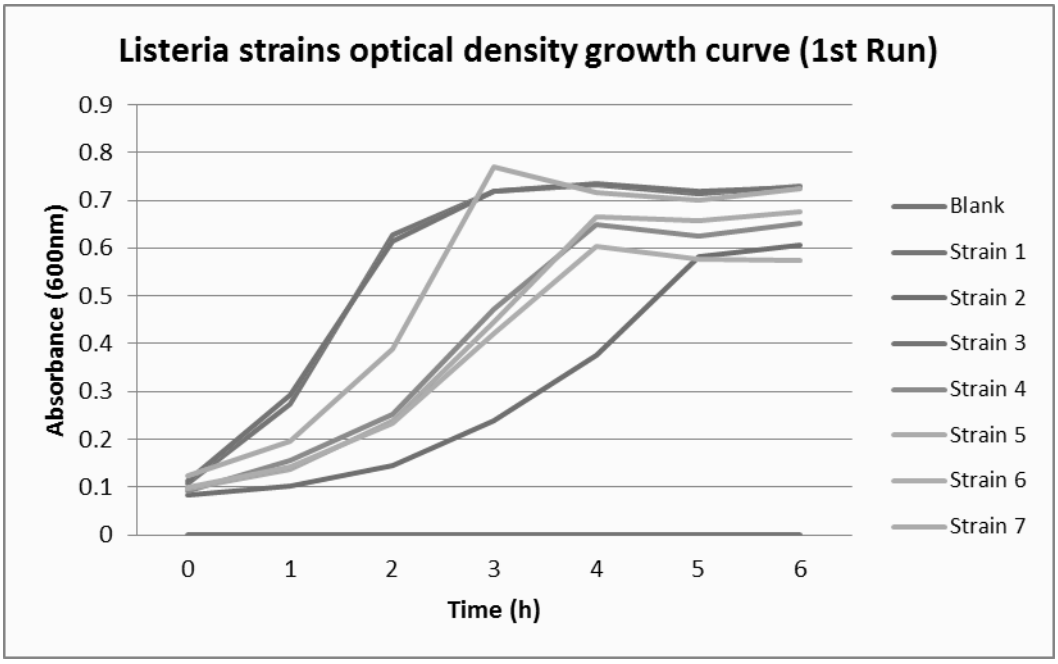


Figure 2

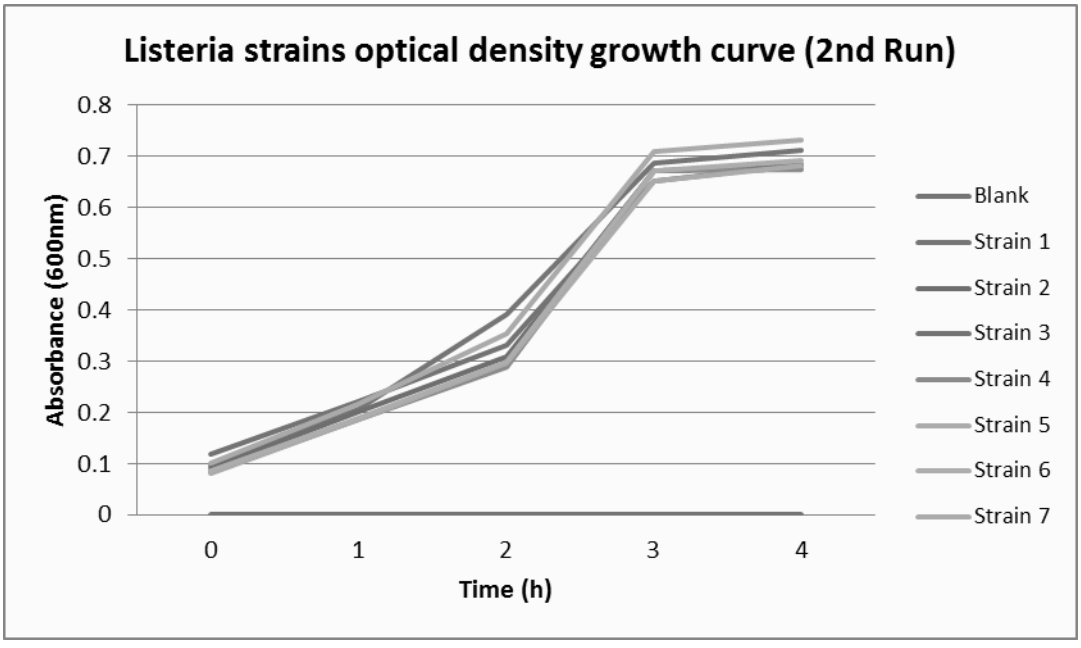


Figure 3

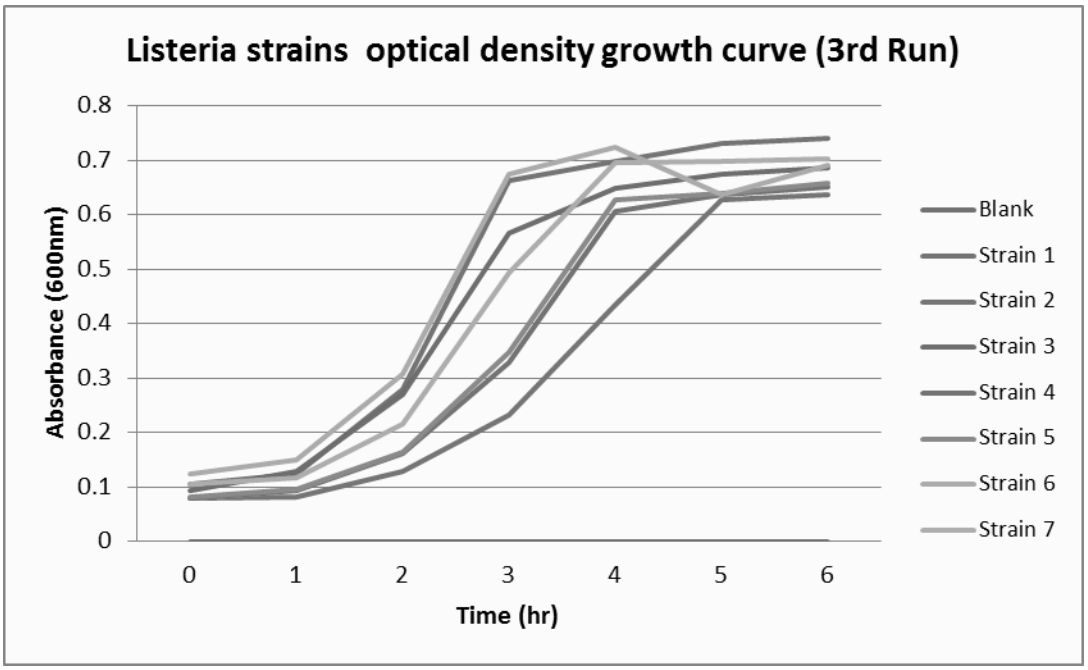


Figure 4

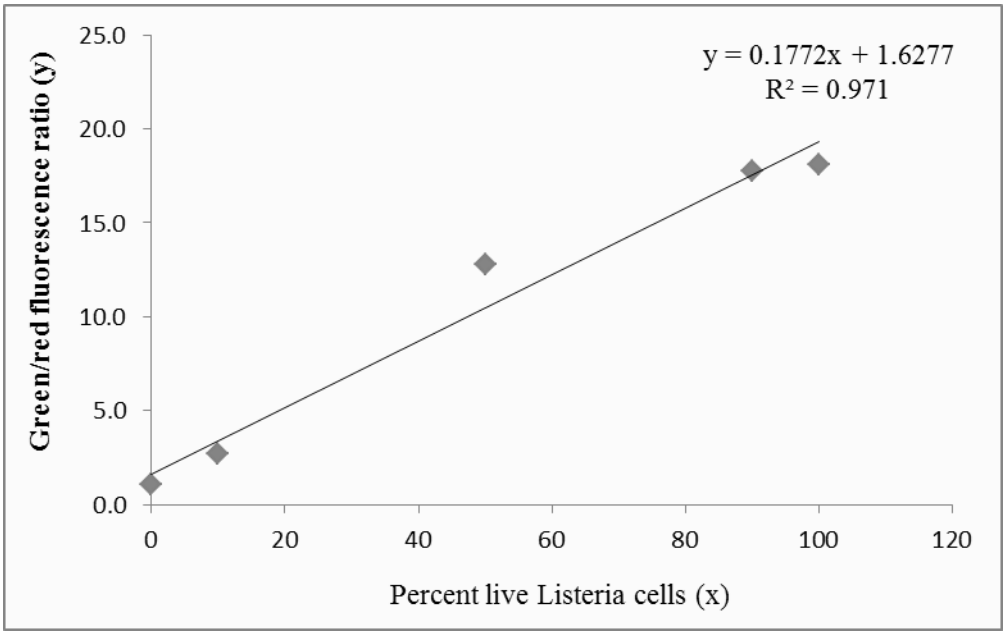
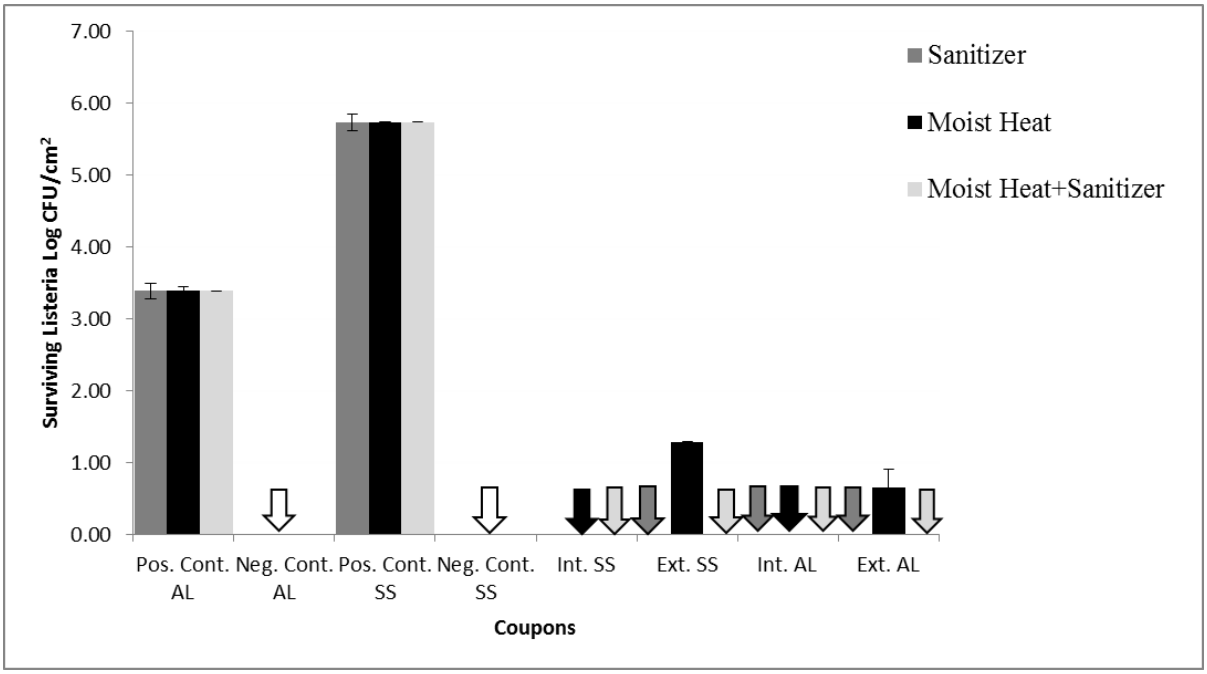


Figure 5


7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Surviving Listeria Log CFU/cm²
Sanitizer
Moist Heat
Moist Heat+Sanitizer
Pos. Cont. AL
Neg. Cont. AL
Pos. Cont. SS
Neg. Cont. SS
Int. SS
Ext. SS
Int. AL
Ext. AL
Coupons

GENERAL CONCLUSION

This study underscored the importance of using appropriate cleaning materials and hygiene monitoring methods in order to ensure sanitary food contact surfaces in food service facilities. The inability of all the cleaning cloths evaluated in this study to reduce contamination to acceptable levels emphasized the need to use cleaning cloths with sanitizing agents or disinfectants. The study further demonstrated that the TPC and ATP-B hygiene monitoring methods could be used interchangeably as they yielded comparable trends of surface contamination levels. Furthermore, the ability of the ATP-B test to generate real-time results could assist food-service establishments by providing quick verifications for total surface cleanliness estimates including the presence of organic fragments and microbial contamination. However, the use of ATP-B should not replace the TPC method but should be used as a complementary method to provide quick confirmation of bacterial contamination on food contact surfaces.

Inefficient cleaning of food contact surfaces could expose consumers to harmful pathogens resulting in serious public health threats. This study has demonstrated that moist heat and moist heat + disinfectant treatments at 150 °F (66°C) and at least 20% RH for 5 h is adequate to attain non-detectable levels of a *Listeria* strains cocktail inoculated on stainless steel and cast aluminum deli meat slicer components. Inactivation of *Listeria* cells inside the motor compartment of the deli meat slicer demonstrated the ability of moist heat to reach even those harborages that may not be easily accessible with traditional cleaning and sanitizing methods. The combination of moist heat with a disinfectant enhanced the inactivation of *Listeria* strains. A special moist heat oven, designed for thermal inactivation of *Listeria* strains, or the deli bread proofer oven, operating at the parameters used in this study, may provide additional protection against cross-contamination with *Listeria* strains. This treatment would have to be carried out after a thorough cleaning and disinfection of the deli meat slicer in order to provide a better margin of safety against pathogenic *Listeria* strains.

It was demonstrated, through the BacLight bacterial viability test, that the moist heat treatment applied in this study was effective in inactivating *Listeria* strains as shown by only less than 4% live cells detected per coupon in some treated coupons. The study also showed that the sub-lethal temperature used in this study could induce the VBNC state in *Listeria* strains as demonstrated by the absence of growth on nutrient agar plates and detection of live cells by the viability test. However, as already mentioned, the proportion of detected live cells was less than 4% per coupon, as shown in Table 1, which might suggest the effectiveness of these treatments. The results of this part of the study are still preliminary, and more experiments still need to be carried out to ensure more accurate results.